Applicant Remarks

In this after-final response Applicant addresses three key points that establish and demonstrate the patentability of the instant claims. First, with respect to the rejection of claims under 35 U.S.C. 112, second paragraph, Applicant has amended the claims to more clearly and distinctly claim the subject matter which applicant regards as the invention. Second, with respect to rejection under 35 U.S.C. 102 (b) as being anticipated by Morimoto et al, Applicant demonstrates that, contrary to Examiner's argument in the final office action, the use of either denatured or undenatured proteins as ingredients necessarily results in different products, even if the proteins in each product are ultimately denatured. Third, with respect to the rejection of claims under 35 U.S.C. 103(a) as being unpatentable over Feldbrugge et al, Applicant shows the products of Feldbrugge et al and of the instant claims are not obvious variants of each other, due to the fact Feldbrugge et al does not disclose a product formed from a composition containing substantial amounts of undenatured proteins, and due to the fact Feldbrugge et al specifically teaches away from the use of an exit die. The inclusion of substantial amounts of undenatured protein and use of an exit die are two key components of the instant claims, as currently amended. Applicant respectfully requests Examiner reconsider the rejection of the claims in light of the amendments and arguments submitted herein.

1. Response to rejection of claims 1-6, 13-26, 56-61, 68-81, 109, and 111-119 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In the current office action, with respect to independent claims 1, 56, and 109, Examiner has stated "it is not clear if the percentages cited therein refer to the final textured whey protein product or whether they refer to the composition prepared prior to the thermoplastic extrusion." In order to provide the level of clarity requested by Examiner, Applicant has amended independent claims 1, 56, and 109 to include the phrase "said composition comprising..." Applicant believes the amendment of the independent claims should be sufficient to overcome the rejection of each claim rejected under 35 U.S.C. 112, second paragraph. Applicant believes the amendment clarifies the percentages cited in independent claims 1, 56, and 109 refer to the

composition prepared prior to the thermoplastic extrusion. If the language of the instant claims does not provide Examiner's desired level of clarity, Applicant is open to any suggested language that would meet the requested level of clarity.

Applicant also wishes to note claim 111 has been amended to depend from claim 109 and is no longer dependent on cancelled claim 110. Applicant thanks Examiner for the kind examination of claims 111-114 as though they depended on claim 109 and believes the current amendment should overcome the rejection of claims 111-114 as indefinite due to dependency on cancelled claim 110.

2. Response to rejection of claims 1-4, 13-16, 56-59, 68-71, 109 and 111-113 under 35 U.S.C. 102 (b) as being anticipated by Morimoto et al.

Under 35 U.S.C. § 102, anticipation requires that each and every element of the claimed invention be disclosed in a prior art reference. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1554, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984). In addition, the prior art reference must be enabling, thus placing the allegedly disclosed matter in the possession of the public. *In re Brown,* 329 F.2d 1006, 1011, 141 USPQ 245, 249 (CCPA 1964). *Akzo N.V. v. U.S. Int'l Trade Comm'n,* 1 U.S.P.Q.2d 1241, 1245 (Fed. Cir. 1986).

Morimoto et al discloses a texturized protein product comprising a thermoplastic extruded product of a mixture comprising a dried heat-coagulable protein and starch. Examiner states the heat-coagulable protein is "inherently food-grade and undenatured, at least until heat treated," however, the denaturation prior to inclusion in the extruder will unavoidably result in a different product than is defined by the instant claims. It is clear that the product produced by the process disclosed in Morimoto et al is dependent upon the inclusion of a denatured heat-coagulable protein, whereas the product produced by the process disclosed by Applicant is dependent upon the inclusion of undenatured heat coagulable protein; therefore, Applicant respectfully requests Examiner to consider the fact the difference in the state of the protein ingredient used in extrusion would inherently and unavoidably lead to products with unique characteristics. In other words, it is not the mere presence or absence of denatured protein that

characterizes the products of Morimoto et al and of the instant claims, but, rather, it is the nature of how the protein ingredients in the compositions of Morimoto et al and of the instant claims interact with the other ingredients and are incorporated into the final products that accurately characterize the final products.

The introduction of Onwulata et al discusses the scientific facts governing protein behavior during the extrusion process. For Examiner's convenience, Applicant has included a copy of the Onwulata et al reference at the end of this office action reply. Specifically, in paragraph two of the introduction section of Onwulata et al, it is shown that in the technical field of food extrusion, it has long been known that the source, concentration, and processing conditions of the starting ingredients are significant factors in determining the molecular behavior of whey proteins during extrusion. With respect to protein behavior during extrusion, a partial or complete denaturation prior to extrusion (as disclosed in Morimoto et al) obviously affects their structure and leads to changes in crosslinking and complexing of the whey proteins during extrusion; therefore, proteins denatured prior to addition to an extruder will alter the nature of the ingredient matrix and functionality. In the first paragraph of their conclusion, Onwulata et al even refer to thermally denatured whey protein isolate as a "unique" ingredient. The unique molecular natures of denatured and undenatured protein is a scientific fact, true now and true at the time Applicant filed the application of the instant claims. A simple everyday analogy is the inclusion of raw (undenatured) egg in an ingredient mixture for baking a cake. One would certainly not anticipate that inclusion of scrambled eggs (precooked and denatured) would result in a product similar to cake made with raw eggs, because the essential molecules (mostly proteins and fats) of the cooked egg cannot produce the same molecular interactions or final product that the uncooked egg produces.

The present patent application is a continuation-in-part of application No. 09/596,191, filed on June 16, 2000, now Pat. No. 6,607,777. Although Onwulata et al did not publish until 2003, the second paragraph of the introduction of Onwulata et al clearly demonstrates that at the time of Applicant's invention, one in the art would have recognized that denatured and undenatured protein behave differently, as discussed above. The second paragraph of Onwulata et al reads as follows:

> The source, concentration, and processing conditions of the starting whey proteins are significant factors, since partial or complete denaturation affects their structure and leads to changes in crosslinking and complexing of the whey proteins during extrusion (Aboagye and Stanley, 1987). Proteins can be pretexturized or denatured before adding to the extruder to create a better matrix, which could lead to improved functionality (Kester and Richardson, 1983; Mohammed et al., 2000).

Kester and Richardson, 1983, is the reference cited by Onwulata e al that most directly pertains to the question of whether or not denatured and undenatured proteins have different potential in terms of functionality and production of specific products (for Examiner's convenience, Applicant has included a copy of the Kester and Richardson article at the end of this office action reply). If desired, full description of the other articles cited by Onwulata et al can be found in the references section of Onwulata et al. In the Introduction of Kester and Richardson, line 14, the effect of the pretreatment of whey, as disclosed by Morimoto et al, is discussed:

> Nutritional value of whey protein concentrates is excellent. Functional properties can be variable, depending upon the source and pretreatment of the whey, extent of protein denaturation, and the presence of nonprotein components.

It is clear that denatured and undenatured proteins are not equivalent ingredients and are not predicted to, and in fact do not, give rise to identical products; therefore, the product claimed by Morimoto et al is necessarily not the same as the product of the instant claims of the Applicant. Morimoto fails to disclose each limitation of the instant claims and Applicant respectfully requests Examiner withdraw the rejection of claims 1-4, 13-16, 58-59, 68-71, 109 and 11-113 under 35 U.S.C. 102(b).

3. Response to rejection of claims 1-6, 13-16, 23-26, 56-61, 68-71,77-81, 109, 111-113, and 119 under 35 U.S.C. 103(a) as being unpatentable over Feldbrugge et al et al.

Pursuant to established legal authority, patentability under 35 U.S.C. § 103 requires a four-step factual analysis, which involves (1) determining the scope and content of the prior art, (2) ascertaining the differences between the prior art and the claimed inventions, (3) resolving the level of ordinary skill in the pertinent art, and (4) utilizing the objective evidence of nonobviousness that may have been presented. *Graham v. John Deere Co.,* 383 U.S. 1, 17, 148 USPQ 459, 467 (1966). After all of these factors have been considered, the ultimate legal conclusion on the issue of obviousness must be reached. With the above background in mind the rejections under 35 U.S.C. § 103(a) will be discussed.

Applicant has previously argued that Feldbrugge et al discloses dense, substantially unpuffed, fibrous products that simulate the muscle of animals or the flesh of fish. (Abstr.) These fibrous products are made by "feeding a proteinaceous dough through a heated channel of decreasing volume to simultaneously elongate and thermally coagulate the dough and then releasing the compression *without forcing the dough through a die* while maintaining a pressure drop below 100 psi." (Abstr. emphasis added) More particularly, Feldbrugge et al used a Sigma mixer to mix ingredients prior to extrusion in a single screw extruder. (Col. 7, lines 30-31; col. 7, line 67, through col. 8, line 2; col. 8, lines 14-17; col. 8, lines 55-56; col. 9, lines 2-4.). Applicant wishes to emphasize the product of Feldbrugge et al can only be produced by a process that does not use an exit die (Abstr.; col. 2, lines 17-19; col. 3, lines 27-32;col. 7, lines 20-23; also col. 3, line 45). Clearly, Feldbrugge teaches away from a product produced with an exit die. Applicant has amended independent claims 1, 56, and 109 to require the use of an exit die, as disclosed in Applicant's specification (see Patent Application Publication US 2004/0161519 A1, specifically paragraph 0049, lines 1-5, paragraph 0049, and paragraph 0073). Feldbrugge et al also teaches the products claimed and disclosed in Feldbrugge et al CANNOT be made by a process that uses an exit die (see col. 3, line 28); therefore, the products of Feldbrugge et al and of the instant claims are clearly not the same. Furthermore, nothing in Feldbrugge et al suggests the use of an exit die to produce an alternative product similar to applicants – this is because Feldbrugge et al discloses that a useful product cannot be produced with the use of an exit die. It is clear Feldbrugge teaches away from the use of an exit die, therefore; Applicant respectfully requests withdrawal of the rejection of claims 1-6, 13-16, 23-

26, 56-61, 68-71, 77-81, 109, 11-113, and 119 under 35 U.S.C. 103(a) as being unpatentable over Feldbrugge et al et al.

Applicant also requests Examiner reconsider the issue of the inclusion of "at least about 25% by weight of undenatured whey protein" in the independent claims 1, 56, and 109. In the current office action Examiner states that Feldbrugge et al et al discloses that a "certain amount" of the protein employed must be undenatured. In fact, Feldbrugge et al discloses a requirement for only "a minimum percentage of undenatured protein" (see col. 3, line 64), not a "certain amount." Applicant respectfully argues that the term "a minimum percentage" is common in many arts and is almost always used to describe some minor component of a mixture (almost universally < 5%). The term "a minimum percentage" does not generally disclose the inclusion of higher percentages of a particular ingredient or component of a mixture, and Feldbrugge et al makes no mention of inclusion of undenatured protein beyond the minimum percentage required. The key characteristic of whey protein in food extrusion is that it is heat coagulable. As far back as 1975, Sternberg et al (a copy of which in included in this reply) established that the minimum heat coagulable, or least coagulable protein concentration, for whey protein varies from approximately 1% to 4.8 % (see figure 3 and the last paragraph of page 1047). As argued above, in the technical field of food extrusion it is well known that the source, concentration, and processing conditions of the starting ingredients are significant factors in determining the molecular behavior of whey proteins during extrusion. With respect to protein behavior during extrusion, a partial or complete denaturation prior to extrusion obviously affects their structure and leads to changes in crosslinking and complexing of the whey proteins during extrusion; therefore, proteins denatured prior to addition to an extruder will alter the nature of the ingredient matrix and functionality. The inclusion of some "minimal percentage" of undenatured protein by Feldbrugge et al does not produce the same product as the instant claims, and, importantly, the teaching of Feldbrugge et al do not suggest the inclusion of higher levels of undenatured protein and cannot be said to predict the product of the instant claims, due to the complexity of molecular interactions of proteins and other ingredients. Furthermore, Feldbrugge provides no reason or motivation for inclusion of undenatured protein levels at or near 25% by weight of undenatured whey protein.

Given the difference between the product produced by the method of Feldbrugge et al and the product produced by the method of the instant claims, Applicant respectfully requests

Examiner reconsider the second and third components of the *Graham* factors. Due to the presence or absence of an exit die and significant difference in percentages of undenatured protein included, Applicant respectfully argues that it cannot be said that the product of the process of the instant claims is rendered obvious by Feldbrugge et al. Specifically, there is nothing in the prior art to bridge the gap between what is disclosed in Feldbrugge et al and the product of the instant claims. Applicant respectfully requests the rejection under 35 U.S.C. 103(a) as being unpatentable over Feldbrugge et al, be withdrawn.

4. Response to rejection of claims 5, 6, 60, and 61 are rejected under 35 U.S.C. 103(a) as being unpatentable over Morimoto et al alone or in view of de Ruyter,

and

Response to rejection of claims 23-26 and 78 – 81 under 35 U.S.C. 103(a) as being unpatentable over Morimoto et al.,

and

Response to rejection of claims 17, 18, 72, 73, 114, and 115 under 35 U.S.C. 103(a) as unpatentable over Morimoto et al or Feldbrugge et al taken together with Villagran et al.,

and

Response to rejection of claims 19-21, 74-76, and 116 - 118 under 35 U.S.C. 103(a) as unpatentable over Morimoto et al or Feldbrugge et al taken together with JP 58-28235, Yackel, Jr. et al, or Ohyabu et al.,

and

Response to rejection of claims 22, 77, and 119 under 35 U.S.C. 103(a) as unpatentable over Morimoto et al or Feldbrugge et al taken together with Ohyabu et al.

Applicant respectfully requests Examiner reconsider each of the above rejections in light of the arguments for allowance of the independent claims 1, 56, and 109 (and therefore all claims depending therefrom), presented in the first three sections of this response.

5. With respect to Examiner's comment that "it should be noted that if it is shown that Morimoto et al and Feldbrugge et al do not provide a whey protein that is undenatured, same would be avoided due to the acid treatment as taught in Ohyabu et al which imparts heat

resistance to the material." The method of Ohyabu teaches treating a finished product in an acid bath in order to stabilize the product, and in no way relates to the specific product formed by the extrusion process disclosed by Applicant. No rejection related to the role of undenatured protein in the formation of the product of the instant claims is justified and no such rejection has been made by Examiner. Furthermore, the disclosure of Ohyabu et al is unrelated to protein extrusion and there is no indication in the art that the process of Ohyabu would be beneficial to protein extrusion products.

Summary

For the reasons presented above, Applicant argues that all current rejections of the instant claims cannot be reasonably sustained; therefore, Applicant requests Examiner withdraw finality and immediately allow all claims.

Respectfully submitted,

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2. Onwulata, C. I., Konstnace, R. P., Cooke, P. H., and Farrell, Jr., H. M. 2003. Functionality of extrusion – textured whey proteins. J. Dairy Sci. 86:3775-3782.
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Modification of Whey Proteins to Improve Functionality

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ABSTRACT

Modification of whey proteins to enhance or alter their functional properties may increase food applications. Whey protein modification can be accomplished by chemical, enzymatic, or physical techniques. Acetylation, succinylation, esterification, amidation, phosphorylation, and thiolation are chemical modifications that can induce significant alterations of the structure and functional behavior of whey proteins. Enzymatic protein modification may entail partial proteolytic hydrolysis, incorporation of crosslinks within the protein conformation, or attachment of specific functional groups to the protein. Physical techniques to alter whey protein functionality include thermal treatment, biopolymer complexing, and texturization.

INTRODUCTION

Annual production of whey resulting from manufacture of cheese in the United States is approximately 20.9 billion kg (129). An estimated 40% of whey is disposed of as waste effluent, resulting in a loss of food energy as well as in a major economic burden. Utilization of surplus whey solids is possibly the most serious problem currently facing the dairy industry (70). Protein accounts for about 15% of the total whey solids and can be recovered via ultrafiltration, gel filtration, complexing with polyphosphates or biopolymers, electrodialysis, or heat/acid precipitation (91, 109). Nutritional value of whey protein concentrates is excellent (24). Functional properties can be variable, depending upon the source and pretreatment of the whey, extent of protein denaturation, and presence of nonprotein components (91).

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To increase applicability of whey proteins in the food industry, functional properties need to be manipulated such that functionality matches the intended use. This can be accomplished by blending, purification, or modification procedures (24). Modification of whey proteins may enhance or alter the combination of functional characteristics, allowing for development of a variety of protein components with a broad spectrum of functional properties. These modified whey proteins may prove useful in the expanding area of fabricated foods.

Protein functionality is governed by physicochemical characteristics of protein, interactions with other protein and nonprotein components, and environmental conditions of the food system. Physicochemical characteristics include molecular weight, amino acid composition and sequence, conformation, net surface charge, and effective hydrophobicity. Modification of a protein to enhance or change its functional performance involves intentional alteration of one or more of these properties. In addition to imparting desired functionality, other objectives in modifying proteins may include an improvement of nutritional value, prevention of deteriorative reactions (e.g., Maillard reaction), or a change in physical state (e.g., texturization) (25, 26).

Modification can be accomplished by chemical, enzymatic, or physical means. Chemical modification entails derivatization of certain amino acid side chains. The objective of chemical derivatization is to alter the noncovalent forces determining protein conformation in a manner that results in desired structural and functional changes. Noncovalent forces of importance in terms of influencing protein conformation include van der Waals forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonds (112). Enzymatic modification generally involves proteolytic hydrolysis of the protein to yield a mixture of peptides. Functional behavior usually is altered in a manner dependent upon

the extent of hydrolysis. Enzymes also can be used to introduce intramolecular or intermolecular crosslinks into a protein structure (93, 133). Polymeric products may exhibit interesting rheological and functional characteristics. Physical protein modification may involve thermal treatment, complex formation with biopolymers, or a texturization procedure. Thermal treatment resulting in partial protein denaturation may elicit desired improvements of functional behavior. Complete denaturation, however, usually results in a loss of solubility and other functional properties. Interaction between proteins and certain hydrocolloids may produce a complex with unique functional properties. Texturization of protein involves physical treatments such as fiber spinning or thermoplastic extrusion. These processes impart structural integrity to proteins.

CHEMICAL MODIFICATION

Relatively small alterations of structure, brought about through chemical derivatization, often can be reflected in significant changes of physical and biological properties (25). Chemical modification has been an effective tool for fundamental investigations of protein conformation and enzymatic mechanisms (53, 88); however, chemical treatment of food proteins to alter functionality has been studied only to a limited extent.

There are many functional groups on amino acid side chains available for chemical derivatization (Table 1). An overview of the various types of protein derivatization reactions recently has been published (26). The susceptibility of a single amino acid side chain to modification is dependent upon the inherent reactivity and accessibility of the side chain functional group, reaction conditions employed, properties of the modifying agent, and polarity and charge of neighboring amino acid side chains (25, 26). The majority of food protein structure-function studies have dealt with derivatization of the ϵ amino group of lysine although modification of the ω carboxyl groups of aspartyl and glutamyl residues recently has been investigated (83, 85).

Derivatization of the positively charged amino group or the negatively charged carboxyl group has profound effects on characteristics of protein. Most notably, the surface charge and hydrophobicity are altered. Changes of these properties may result in a modified isoelectric point and conformation, which in turn, influences overall functional behavior of the protein. Specific functional characteristics that can be affected by chemical modification include solubility, surfactant properties, degree of hydration, tendency for gelation, and thermal stability.

Acylation

Acetylation and succinylation of proteins with acid anhydrides are common techniques for chemical modification. Proteins that have been acylated to alter functionality include casein (16, 130), fish protein (14, 36, 37, 130), single cell protein (76), soy protein (29, 130), gelatin (130), leaf protein concentrate (30), pea protein isolate (58), wheat flour protein (33), and whey protein concentrate (126). Under the usual reaction conditions, the ϵ amino group of lysine is acylated most readily.

Acetylation with acetic anhydride involves covalent attachment of neutral acetyl functions to the protein amino group. This results in a partial unfolding of the protein backbone because of reduced electrostatic attraction between oppositely charged amino acid side chains. Practical effects of acetylation may involve a slight increase of aqueous solubility, reduced isoelectric point, and decreased tendency to gel upon heating (67).

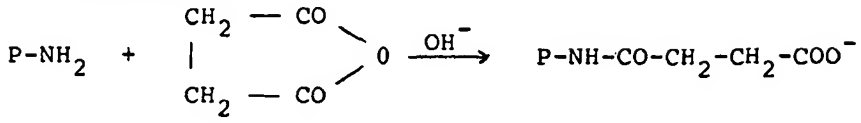
Reaction with succinic anhydride (Figure 1) introduces anionic succinate residues covalently

TABLE 1. Amino acid side chains available for chemical modification.[a]

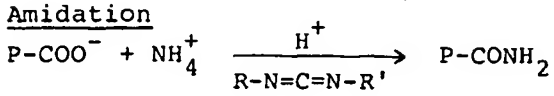
Side chain	Commonly used modifications
Amino	Alkylation, acylation
Carboxyl	Esterification, amidation
Disulfide	Reduction, oxidation
Imidazole	Oxidation, alkylation
Indole	Oxidation, alkylation
Phenolic	Acylation, electrophilic substitution
Sulfhydryl	Alkylation, oxidation
Thioether	Alkylation, oxidation

[a]From reference (25). Copyright 1977, American Chemical Society.

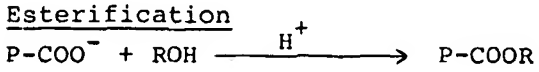
1. Succinylation



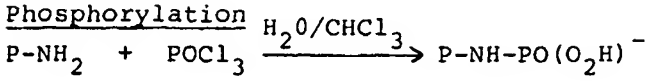
2. Amidation



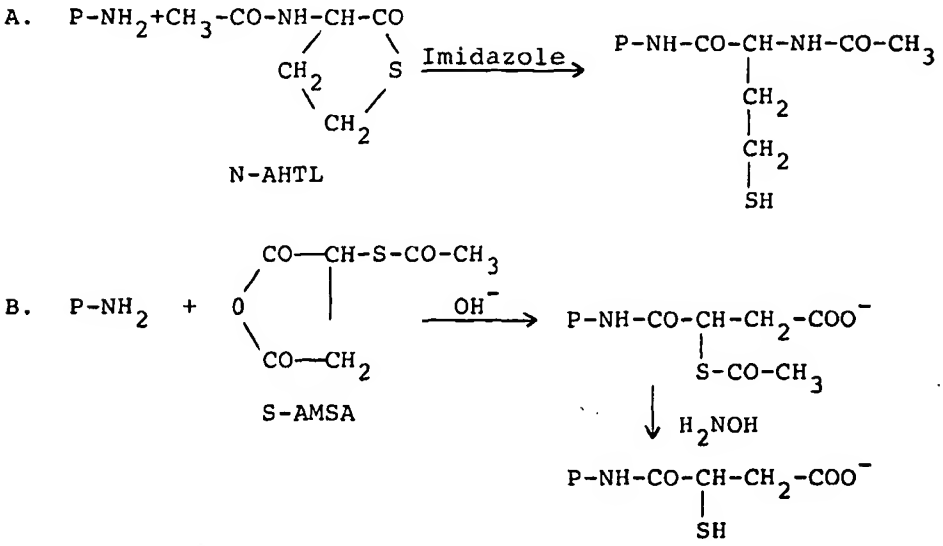
3. Esterification



4. Phosphorylation



5. Thiolation



6. Reductive Methylation

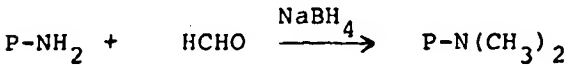


Figure 1. Reactions for the chemical modification of proteins.

linked to the ϵ amino groups of lysine. Succinylation generally has greater effects upon protein conformation and functional behavior than acetylation. The electrostatic repulsive forces, resulting from the enhanced negative charge, lead to more extensive unfolding of the

polypeptide chain. Alterations of functionality commonly associated with succinylation include increased aqueous solubility, enhanced hydration, and modified surfactant properties (29, 67). Improved solubility is due to the ease with which water molecules can penetrate the partially unfolded protein structure. Because emulsification and foaming activities are dependent upon high aqueous solubility, succinylation generally leads to improvements of these properties as well (14, 29, 30, 58, 76).

The effect of acylation on physicochemical properties of whey proteins has not been studied to a great extent. Habeeb et al. (39) investigated structural effects of succinylation on β-lactoglobulin and bovine serum albumin. They noted a significant increase of intrinsic viscosity upon modification, indicative of unfolding or expansion of the protein structures. Electrostatic repulsion produced by the introduction of anionic succinate groups was responsible for the conformation change. In addition, one would expect that the monomer-dimer equilibrium of β-lactoglobulin would be affected by enhanced charge repulsions.

More recently, functional attributes and food applications of succinylated, heat denatured-acid precipitated whey protein have been examined (124, 125, 126). Isolation of whey protein concentrate via heating acidified whey, to denature and precipitate the protein, results in a water-insoluble powder, commonly referred to as lactalbumin (109). Except for the ability to bind water, lactalbumin exhibits extremely poor functionality, which limit its scope of food applications. Upon reaction with succinic anhydride at pH 8, extensive unfolding of the protein was observed, resulting in improved functional properties (126). Succinylation of 77% of the available amino groups improved solubility at pH higher than the isoelectric point (approximately pH 4); however, solubility at pH 7 was still only 24%. Water absorption was increased 4 to 5 times; the modified (77%) product absorbed 13 times its weight in water. Effects of succinylation on surfactant properties were variable. Whipping properties were rendered inferior, but oil emulsifying capacity and stability markedly improved with degree of succinylation. Emulsion capacity and stability of the succinylated (77%) lactalbumin were significantly greater than for a commercial soy protein isolate and a commercial whey protein concentrate isolated via ultrafiltration. Viscosity of 5% protein dispersions increased upon succinylation.

Succinylated lactalbumin subsequently was evaluated in several practical food applications, including coffee whitener, salad dressing, and as an extender or protein supplement in meat products (124, 125). Replacement of sodium caseinate with succinylated (77%) lactalbumin in a coffee whitener formulation resulted in a more viscous product with comparable emulsion stability and no significant difference in sensory acceptability from the caseinate control. Partial substitution of egg yolk with succinylated lactalbumin in a salad dressing (20% replacement) similarly produced a more stable product with no significant difference in overall acceptability compared to the unsubstituted dressing. Evaluation of succinylated lactalbumin in meat patties demonstrated its potential use as an extender (125). Patties extended with succinylated protein (5 or 10% addition) exhibited increased yield following cooking, greater moisture and fat retention, and no significant difference in appearance, texture, or flavor compared to the all-beef patties. Performance of the modified lactalbumin as a protein supplement in a comminuted meat system was less than desirable. The supplemented product exhibited lower emulsion stability, higher cooking loss, and a lighter, less acceptable color compared to the all-meat control. The depressed emulsion stability is surprising in light of the greater stability upon succinylation in a model system (126), as well as in the coffee creamer and salad dressing applications (124). However, this underscores the influence of other ingredients upon protein functionality in actual food systems.

Results of studies that have been cited illustrate the potential improvement of overall functional behavior of whey proteins, especially heat-denatured lactalbumin, upon acylation. Acylated whey proteins may be applicable to foods requiring high emulsifying activity, water binding capacity, and viscosity.

Amidation/Esterification

A large percentage of proteins have isoelectric points close to pH 5 and are negatively charged at neutral pH (78). Negative charge can be

reduced effectively by chemical derivatization of the free carboxyl groups of aspartate and glutamate residues (82). Change of net charge from negative to positive at pH commonly encountered in foods results in a unique distribution of electrostatic interactions within the protein structure. These new interactions may enhance or alter functional behavior. For amino acid bioavailability, modification of nonessential glutamyl and aspartyl residues may be more acceptable than derivatization of lysine.

Carboxyl groups can be blocked by amidation (25, 73, 77, 83) or esterification reactions (25, 28, 83, 120, 134) (Figure 1). Amidation may be accomplished via a carbodiimide-mediated condensation of carboxyl groups with ammonium ion, effectively converting aspartate and glutamate residues to asparagine and glutamine, respectively. Esterification of carboxyl groups proceeds readily in an alcoholic-HCl medium.

Effects of amidation and esterification of β-lactoglobulin have been examined preliminary to modification of whey protein concentrate (83, 84, 85). The isoelectric point of β-lactoglobulin was increased upon modification. Amidation blocked 78% of available carboxyl groups, producing a modified protein with an isoelectric point of 10.0 compared with 5.2 for native β-lactoglobulin. Esterification with methanol or ethanol produced derivatives with 83 and 55% of the carboxyl groups modified, yielding isoelectric points of 9.8 and 7.2. The effect of enhanced positive charge on conformation of β-lactoglobulin was studied by circular dichroism. An increase of random configuration was evident for both amidated and esterified protein and was indicative of partial denaturation.

The change of net charge and conformation upon amidation or esterification of β-lactoglobulin was reflected by profoundly different functional properties. These positively-charged derivatives exhibited strong electrostatic interaction with casein micelles of bovine milk (84). Increasing modified protein caused a progressive reduction of surface charge repulsion between micelles, resulting in a dramatic decrease of rennet coagulation time (Figure 2). It was estimated that 1.0 g of amidated, 2.0 g of ethyl-esterified, and 1.0 g of methyl-esterified β-lactoglobulin would coagulate 100 ml of casein micelles at concentrations in milk without addition of rennet extract (84). Coagulation of skim milk mediated by positively charged proteins also has been reported by other investigators (21, 35).

Emulsifying activity of amidated and esterified β-lactoglobulin was slightly lower than that of native protein (85). However, stability of emulsions prepared with the ethyl-esterified derivative was significantly greater than emulsions prepared with native β-lactoglobulin or other modified proteins. Over 40% of the ethyl-esterified protein adsorbed to the interface of oil and water, almost four times more than other proteins, perhaps explaining the increased emulsion stability. The authors interpreted the unique oil adsorption of the ethyl derivative to be a possible consequence of lower isoelectric point. Average net charge at neutral pH is less than the other β-lactoglobulin derivatives, which may favor increased association at the interface of oil and water. Furthermore, attachment of the more hydrophobic ethyl residues also may be responsible for the unusual adsorption. In any event, the unique interfacial behavior of this esterified whey protein merits closer examination.

The positive charge of amidated and esterified whey proteins, combined with the remarkable

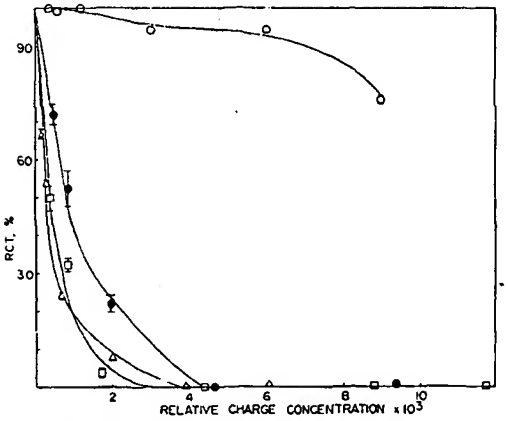


Figure 2. Percentage decrease in rennet coagulation time (% RCT) of casein micelles after addition of increasing concentrations of indicated proteins as compared to RCT of untreated micelles (84). Relative charge concentrations (±) of native β-lactoglobulin (○), amidated β-lactoglobulin (●), ethyl-esterified β-lactoglobulin (△), or methyl-esterified β-lactoglobulin (□) were calculated according to the method of Green and Marshall (35). n=3. Reported as ± SD.

interfacial properties of the ethyl-esterified β-lactoglobulin, suggest potential application in the cheese industry as electrostatic coagulants, or in food systems that require high emulsion stability.

Phosphorylation

Phosphate groups can be attached covalently to proteins by an assortment of techniques (Figure 1), reaction with phosphorus oxychloride being the most widely used (12, 44, 81, 86, 108, 113, 135, 136). Alternative reagents that have been utilized include cyclic sodium trimetaphosphate (123), phosphorus pentoxide dissolved in phosphoric acid (20, 27), phosphoramidate (94, 104), and phosphoric acid combined with trichloroacetonitrile (128, 139). Chemical phosphorylation of proteins may involve derivatization of the hydroxyl oxygen of serine and threonine residues (27, 81, 123, 128, 135) or the amino and imidazole nitrogen of lysine and histidine (81, 86, 104, 123, 136). In addition, reaction with phosphorus oxychloride apparently generates crosslinks within the protein structure (81, 136). The nature of the crosslinks is unknown; however, they may be phosphate bridges (81) or isopeptide linkages (136).

Because protein surfaces are already relatively hydrophilic, introduction of phosphate groups may not exert as dramatic an effect on functionality as attachment of hydrophobic groups. Nevertheless, variations of the manner in which protein and water interact could be expected. Altered interaction of protein and water may influence solubility, water absorption, viscosity, gel forming characteristics, and surfactant properties. Elevated viscosity of protein dispersions following phosphorylation was observed in (44, 81, 86). Water absorption reportedly increased upon phosphorylation of casein and lysozyme; however, aqueous solubility decreased (81). Protein crosslinks incorporated through reaction with phosphorus oxychloride was postulated as the reason for depressed solubility. Treatment of soy protein isolate with sodium trimetaphosphate enhanced water solubility, water-holding capacity, and emulsification and whipping properties (123).

Effects of chemical phosphorylation of β-lactoglobulin have been evaluated prior to possible modification of whey protein concentrate (136, 137). Reaction with phosphorus oxychloride at pH 8.5 produced a derivative containing approximately 13 moles of phosphorus per mole of protein. The nitrogen of lysyl and histidyl residues were the principal sites of phosphate attachment. Native conformation of the protein was disrupted partially as revealed by circular dichroism. Dephosphorylation of the modified β-lactoglobulin failed to regenerate the native circular dichroic spectrum, suggesting irreversible changes of secondary and tertiary structure upon phosphorylation.

Functional performance of the phospho-β-lactoglobulin derivative was distinctly different from the native protein (137). Of particular interest was the observation that a 6% (wt/vol) solution of the phosphoprotein readily gelled in the presence of 100 mM Ca^{2+} at pH 5.0 without thermal treatment (Figure 3). The fluid yogurt-like gel presumably formed via calcium crosslinks between negatively charged phosphate groups.

Emulsification properties also were altered upon modification. Forty percent (wt/wt) corn oil emulsions were rendered more stable by as much as 30% when prepared with phospho-β-lactoglobulin as compared with emulsions prepared with native β-lactoglobulin. Improved stability was likely due to a reduced isoelectric point for the phosphoprotein. The enhanced negative charge caused greater repulsion between protein enrobed oil droplets, preventing coalescence (137). Relative viscosity of 65% (wt/wt) corn oil emulsions prepared with phospho-β-lactoglobulin, and subsequently exposed to 100 mM Ca^{2+}, was more than double that of a similar emulsion containing native β-lactoglobulin. The 65% emulsion prepared with the phosphorylated derivative had an apparent viscosity equal to or greater than two commercial brands of mayonnaise. The increased viscosity was ascribed to formation of calcium crosslinks between particles in the emulsion (137).

The improved emulsifying and gel-forming properties achieved upon modification suggest an array of potential food applications for phosphorylated whey proteins. Possible uses exist in foods that require high emulsification activity, such as dressings and mayonnaise.

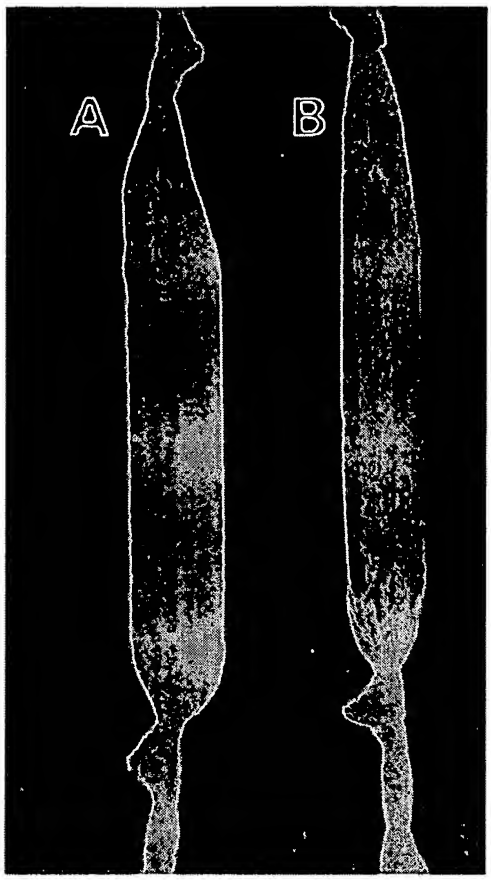


Figure 3. Formation of gel upon exposure of a 6% wt/vol solution of phosphorylated β-lactoglobulin to 100 m*M* Ca^{2+}. A) Phosphorylated β-lactoglobulin, B) native β-lactoglobulin (6% wt/vol) exposed to 100 m*M* Ca^{2+}. Proteins dissolved in .1 *M* acetate buffer, pH 5 (137).

Thiolation

Thiol and disulfide groups are important determinants of functionality in many proteins. They play a particularly vital role in the rheology and structure of wheat flour dough, where the breaking and reformation of disulfide bonds contribute the unique viscoelastic properties (10, 59). Other proteins markedly influenced by sulfhydryl and disulfide groups include bovine β-lactoglobulin and κ-casein, which form a disulfide linked complex upon heating that prevents gelation of sterilized milk (103, 114); egg albumin, of which heat mediated gelation is partially dependent upon sulfhydryl-disulfide interchange (77, 95); and soy protein, in which thiol groups have a role in texture formation during fiber spinning (62).

Functional attributes of food proteins directly or indirectly governed by sulfhydryl groups include elasticity, gelation, texturization, viscosity, and thermal stability. These properties may be altered or extended through appropriate oxidation and reduction reactions resulting in rearrangement of intramolecular and intermolecular disulfide bonds. This restructuring of the protein conceivably could yield a variety of novel monomeric or polymeric species with dramatically different functionality.

Certain physical properties of protein might be modified radically by covalent attachment of new sulfhydryl groups. Chemical thiolation of proteins has been accomplished with several reagents, including polythioglycolides (115), N-acetylhomocysteine thiolactone (N-AHTL) (1, 7, 8, 63, 119), and S-acetylmercaptosuccinic anhydride (S-AMSA) (68, 69). Reaction with N-AHTL in alkaline conditions involves an imidazole catalyzed acylation of the amino groups (Figure 1, reaction 5A) accompanied by opening of the thiolactone ring and exposure of a new sulfhydryl group. Thiolation with S-AMSA is also an acylation of amino groups; however, the sulfhydryl function is protected in the form of an acetylthio group. The acetyl group subsequently can be removed by a nucleophilic displacement with hydroxylamine (Figure 1, reaction 5B).

Thiolation of β-lactoglobulin with N-AHTL and S-AMSA has been reported (64). Reaction with N-AHTL resulted in acylation of more than 90% of the available amino groups, whereas S-AMSA thiolated approximately 80% of the amino groups. As many as 20 new sulfhydryl functions were incorporated per β-lactoglobulin dimer (M_r = 37,000). As would be expected from reduction of lysine amino groups, the isoelectric point, and therefore the net charge at neutral pH, were altered upon thiolation. Acylation with N-AHTL reduced the isoelectric point from 5.2 for native β-lactoglobulin to pH 4.5 to 4.7. The S-AMSA derivatized protein possessed a lower isoelectric point of 3.7 because of introduction of an added free carboxyl group for each added sulfhydryl (65). Potassium iodate was used to oxidize sulfhydryl groups of the thiolated β-lactoglobulin to

disulfide bonds. Both intramolecular and intermolecular disulfide crosslinking occurred, leading to formation of polymeric products with molecular weights exceeding 600,000. These novel β-lactoglobulin polymers exhibited certain unique characteristics, such as enhanced heat stability, increased viscosity, foaming ability, and gel forming ability. In the presence of Ca^{2+} ions, the polymers formed a strong, transparent, heat-stable gel that was irreversible even upon treatment of the gel with ethylenediaminetetraacetate (EDTA) (65). The polymers displayed enhanced thermal stability, as shown by differential scanning calorimetry. The resistance to heat coagulation is probably due to a more random conformation, resulting from disruption of the surface charge upon thiolation. For structure and thermal stability, the thiolated derivatives of β-lactoglobulin seem to be analogous to the caseins, which are considered naturally denatured proteins possessing remarkable stability against heat precipitation.

Reductive Alkylation

Reductive methylation has not been used extensively to improve functionality of food proteins. Most studies suggest methylation exerts minimal effects on protein character. The rationale for including this reaction is its application as an analytical tool for the study of milk protein interactions during handling, processing, and storage of dairy products. Other objectives in attaching methyl groups to a protein without affecting charge, conformation, or overall functional behavior could be to elucidate biological pathways or to block deteriorative reactions. For example, reductive methylation of protein amino groups (32, 74, 87) may be beneficial in preventing the Maillard reaction during processing and storage of carbohydrate-containing foods.

Specific methylation of amino groups can be accomplished via reaction with formaldehyde and sodium borohydride (Figure 1) (87). An alternative procedure utilizes sodium cyanoborohydride at pH 7 in place of sodium borohydride (23, 56). Presumed advantages of using the weaker reducing agent, cyanoborohydride, are the greater efficiency of methylation and elimination of potential side reactions attendant with the use of borohydride at a higher pH (56, 57).

For reductive methylation to be a useful labeling technique, it is essential that the reaction exert only minimal change of the native properties of the protein. The small size of the added methyl functions, combined with the minor alteration in dissociation constant (pKa) of the derivatized amino group, should yield minimal perturbation of protein structure and function (57). Reductive methylation of α_S-, β-, and κ-caseins had little influence on the physicochemical character of the proteins (97). Solubility, electrophoretic mobility, and susceptibility to Ca^{2+} precipitation were either unchanged or altered to a minimal degree. Similarly, methylation of β-lactoglobulin caused only minor changes of protein character (111). Methylation of 80% of the amino groups failed to alter free-sulfhydryl content or electrophoretic mobility, and shifted the isoelectric point only .1 pH unit. Heat denaturation of the methylated β-lactoglobulin was compared to native protein via use of an isotopically tagged derivative prepared with [^{14}C] formaldehyde. When added to skim milk, the tracer [^{14}C] methylated protein displayed a heat denaturation curve virtually identical to that of unmodified β-lactoglobulin (111).

Use of [^{14}C] formaldehyde to label radioactively protein by reductive methylation has been reported by many investigators (23, 56, 57, 98, 105, 110). The high specific activities attained make this a sensitive approach for quantifying protein interactions. This technique recently has been applied to the study of protein interactions in heated skim milk (110), to follow enzymatic hydrolysis of milk proteins (22, 110), as well as to monitor electrostatic interactions between casein micelles and positively charged β-lactoglobulin derivatives (84).

Although studies have suggested that reductive methylation causes insignificant effects upon protein conformation, there are probably subtle changes that become evident only when more sensitive analyses are applied. For example, a recent study using differential scanning calorimetry and circular dichroic spectroscopy revealed that extensive attachment of methyl groups does have an effect on the structure of some proteins (31). In addition, Sen et al. (117) noted a slightly modified

ultraviolet spectra and improved emulsifying activity with highly methylated casein, indicative of a change in conformation.

Reductive alkylation with alkylating reagents of a larger size or different polarity than methyl groups may be a possible technique for achieving more significant effects upon whey protein functional behavior. A study of properties of six highly alkylated casein derivatives, including methyl-, isopropyl-, butyl-, cyclopentyl-, cyclohexyl-, and benzylcasein, revealed that the native conformation of casein was altered to an extent dependent upon the size of the hydrophobic alkyl group (117). The larger the attached alkyl group, the greater the deviation of the ultraviolet absorption spectrum from that of native casein. Except for butyl- and cyclopentylcasein, the highly alkylated caseins exhibited enhanced water absorption, which is surprising considering the hydrophobicity of the incorporated alkyl groups. Emulsifying activity also was improved markedly for the alkylated caseins, with the exception of butylcasein.

The attachment of sugar residues via reductive alkylation (80) represents another potential means of changing whey protein functional performance. As was evident upon phosphorylation of β-lactoglobulin (137), the increased polarity achieved upon glycosylation of a protein may be reflected by modified solubility, viscosity, gelation, and surfactant characteristics. Reductive alkylation in the presence of sodium cyanoborohydride has been used successfully to couple glucose, fructose, maltose, cellobiose, melibiose, and lactose to bovine serum albumin (34, 116) and casein (72).

Covalent Attachment of Amino Acids

Covalent coupling of amino acids to proteins may be a promising technique for improving both nutritive value and physical properties of food proteins. Improvement of nutritional value of whey proteins would be unnecessary; however, the surfactant characteristics might be enhanced by the attachment of hydrophobic amino acid residues to impart greater amphiphilic nature to the proteins.

Amino acids can be incorporated readily into a protein structure by formation of an isopeptide linkage (26, 100). The isopeptide linkage is an amide bond between the carboxyl group of the amino acid to be attached and the ε amino group of lysine of the protein or between the amino group of the incorporated amino acid and a free carboxyl group of the protein. Puigserver et al. (100, 101, 102) successfully attached tryptophan, glycine, alanine, methionine, N-acetylmethionine, aspartic acid, and asparagine to casein. Upon covalent attachment of amino acids to ε amino groups of casein, a conformational change occurred, as evidenced by the ultraviolet absorption difference spectra. Solubility or viscosity of casein were not altered significantly through covalent attachment of either the hydrophobic or hydrophilic amino acids (102). This may be attributed to the fact that the casein initially lacks ordered tertiery structure. Similar modification of ordered globular proteins, such as whey proteins, conceivably could yield appreciable changes of functionality.

ENZYMATIC MODIFICATION

Protein functionality can be altered or extended by enzymatic action to hydrolyze partially the polypeptide backbone, incorporate intermolecular or intramolecular crosslinks, or attach specific groups to the protein (133). There are several advantages of utilizing enzymes to perform desired modifications of protein structure and function. Enzymes are generally specific in terms of reactions they catalyze. Therefore, there is little potential for undesirable side reactions, which, combined with the mild conditions necessary for enzyme catalysis, result in minimal tendency to form toxic by-products. Furthermore, enzymes are effective catalysts at low concentration.

Proteases are the most widely used enzymes for modification of food protein. They hydrolyze selected peptide bonds to promote reduction of molecular weight, possible conformation changes, and enhanced hydrophilicity due to newly exposed amino and carboxyl groups (99). Effective hydrophobicity of certain globular proteins conceivably could be increased as well through exposure of apolar amino acid residues upon limited hydrolysis and subsequent unfolding of the polypeptide chain.

General effects of proteolysis on size, structure, and polarity could result in dramatic

changes of protein functional behavior. Specific properties of the hydrolysate are dependent upon degree of hydrolysis, which is influenced by the specific activity of the protease, physical and chemical character of the protein substrate, and reaction conditions. One would intuitively expect an increase of solubility and a decrease of viscosity as hydrolysis progresses. In addition, proteolysis generally leads to reduced tendency for gelation, increased foam volume upon whipping, decreased foam stability, and enhanced thermal stability. Emulsifying activity may be altered as well; however, the specific effect varies with nature of the substrate (106).

Many food proteins have been subjected to limited proteolysis, including soy (4), rapeseed (45), fish (40, 46), and whey protein concentrate (15, 47, 61, 71, 89, 96). Trypsin has been used to hydrolyze whey proteins in several investigations (15, 47, 61, 89). A comparison with papain and a neutral protease from *Bacillus subtilis* revealed trypsin was the most efficient for solubilization of heat-denatured lactalbumin (89). Complete solubilization of lactalbumin was achieved by digestion with trypsin for 3 h at pH 8 and 55°C (61). The resulting hydrolysate consisted of a mixture of peptides ranging in molecular weight from 500 to 5,000. Hidalgo and Gamper (47) utilized trypsin hydrolysis to render whey proteins stable to precipitation upon thermal treatment. The tryptic hydrolysate remained 80% soluble following 5 min of heating at 134°C. Foaming activity of a whey protein tryptic digest was studied by Cooney (15). A significant increase of foam volume and only a relatively small decrease of foam stability were noted.

A study of whey protein hydrolysis with pepsin and two microbial proteases was reported by Kuehler and Stine (71). Proteolysis was extremely detrimental to emulsifying capacity. Limited hydrolysis, however, produced up to a 25% increase of specific foam volume (ml/g protein) upon whipping. Digestion for 30 to 60 min at 50°C appeared to be the optimal treatment for enhancing foam capacity. Extended periods of hydrolysis resulted in a dramatic decrease of foaming ability (Figure 4). Although specific foam volume was improved upon limited proteolysis, stability of the foams was greatly inferior compared to the untreated control. The authors postulated that the enhanced air incorporation was due to the higher content of polypeptides resulting from limited hydrolysis; however, these polypeptides apparently lack the surfactant properties necessary to stabilize a foam. Addition of carboxymethyl cellulose (CMC) to the hydrolysates prior to whipping improved foam stability. This indicates potential use of whey protein hydrolysate/stabilizer blends as functional food additives.

A critical problem hindering the use of proteases for improving functional behavior is excessive hydrolysis. Although limited proteolysis often may yield a more functional protein, it is difficult to limit the degree of digestion to that which promotes desired performance. Enzymes may be inactivated thermally; however, there is a risk of causing undesirable changes in the protein of interest, such as insolubilization from denaturation (99). A possible alternative for controlling the extent of digestion appears to be the use of immobilized enzymes in continuous reactors. These should allow production of hydrolysates with the desired uniform degree of digestion, therefore, more constant and reliable functional behavior.

Another approach for controlling protease activity has been proposed by Haard et al. (38). These authors suggest the possible use of proteases isolated from marine fish to be used

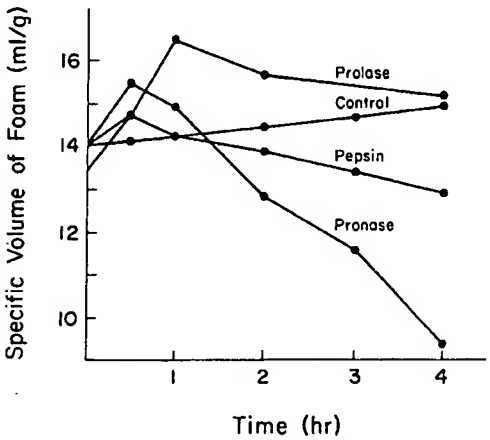


Figure 4. Effect of duration of enzymatic hydrolysis with pepsin and two microbial proteases on specific volume of foam obtained by whipping a heated whey protein solution (71). Reprinted from *Journal of Food Science*. Copyright 1974, Institute of Food Technologists.

as food-processing aids in place of conventional enzymes. The poor thermal stability of fish proteases indicate that these enzymes can be inactivated intentionally by relatively mild heat treatment.

Formation of bitter peptides upon hydrolysis of certain proteins is another problem associated with the use of proteases (2). The tendency for a protein to form bitter peptides is related to the content of hydrophobic amino acids. Casein and soy isolate are examples of proteins with a propensity for developing bitter hydrolysates. Slight bitterness was detected in a trypsin hydrolysate of heat-denatured whey protein, but the off-flavor was not strong enough to prevent use of the hydrolysate in food applications (89).

Use of proteases to increase application of whey protein appears most promising in the area of protein-supplemented beverages. Complete solubility achieved with partial enzymatic hydrolysis will permit whey protein fortification of acidic soft drinks and citrus juices. In addition, the thermal stability of the hydrolysates will minimize solubility loss upon beverage sterilization.

Besides cleaving peptide bonds, enzymes are also potentially useful for incorporation of crosslinks between polypeptide chains or for attachment of functional groups. The feasibility of posttranslational enzyme-catalyzed modifications to enhance the functional and nutritional character of proteins has been reviewed by Whitaker (133). Specificity requirements of many of the enzyme reactions are so strict they limit their potential role in food protein modification; however, certain reactions eventually could achieve importance. For example, enzymatic phosphorylation with protein kinase may be applicable to food proteins (9, 133). Experiments have shown that denatured proteins and proteolytic digests can be phosphorylated in vitro by these enzymes (133). Thus, it appears that the specificity of certain protein kinases is broad enough to make this reaction a possible alternative to chemical phosphorylation.

Thiolation of protein by enzymatic means has been reported (122). A unique papain-catalyzed acylation with N-AHTL was used to introduce new sulfhydryl groups into soy protein. The thiolated derivative had altered solubility, emulsification, foaming, and gelling properties.

Papain also has been utilized to catalyze a plastein type of protein aminolysis resulting in the covalent attachment of methionine ethyl-esters (90, 138) or L-leucine n-alkyl esters to food proteins (3, 118, 130, 131, 132). The latter reaction allows for incorporation of high amphiphilicity, resulting in improved foaming and emulsifying activity. An enzymatic modification of this type may be a viable technique for improving surfactant characteristics of whey proteins, which are not very amphiphilic because of a uniform distribution of apolar amino acids.

Enzymatic crosslinking of whey proteins may be another means of extending their application. Crosslinked protein polymers may possess rheological properties useful in many fabricated food systems. Transglutaminase recently was used to catalyze formation of intermolecular and intramolecular crosslinks in a variety of proteins, including casein and β-lactoglobluin (54, 93).

PHYSICAL MODIFICATION

Changes of protein functional performance through physical means can be achieved by thermal treatment, biopolymer complexing, or texturization. In their native state, whey proteins exist in globular, well-defined, three-dimensional conformations. The majority of food applications require the undenatured form so that solubility, and the functional properties dependent upon solubility, are at their maximum. Heat treatments at about 70°C are critical to physical properties of whey proteins. At or near this temperature, protein solubility, foaming activity, and emulsifying activity start to decline, whereas water binding and viscosity begin to increase (18). More severe thermal treatment will cause protein denaturation accompanied by loss of aqueous solubility and overall functional behavior. Subjecting native whey proteins to thermal conditions that promote only partial denaturation may be a practical way to produce a unique and desirable blend of functional properties. Indeed, partial denaturation, or combining partially denatured with native protein, has been suggested as a technique for intentional modification of functionality (112).

Thermal treatment of whey protein concentrates to initiate partial denaturation has been used to enhance surfactant properties (17, 75, 107). DeWit (17) reported that a preheat treatment between 40 and 65°C is required to ensure good whey protein foaming activity. Richert et al. (107) found that heating whey protein concentrate for 30 min at 60 to 65°C just prior to whipping resulted in reduced whipping time and substantial improvements of foam capacity and stability over the unheated control. The altered foaming activity upon heating has been ascribed to partial unfolding of the whey protein molecules to expose buried hydrophobic amino acid residues. The amphiphilic nature of the protein was enhanced, facilitating proper orientation at the interface of air and water (91).

DeWit's group (19) reported on the novel approach of thermal treatment at pH 7.5 to impart structure-forming characteristics to whey proteins. There is an increase of activity of the sulfhydryl group in this pH range, causing thiol-disulfide interchange reactions as the protein backbone unfolds. This thermal restructuring of whey protein yields improved physical properties resembling those of casein. These properties were demonstrated by preparation of yogurt containing heat-denatured whey protein concentrate in place of casein.

Coagulation with polymers has been studied widely as a method for whey protein recovery. Coagulating agents that have been used include carboxymethyl cellulose (CMC), carrageenan, polyphosphate, sulfonated lignin, polyacrylic acid, and chitosan (11, 13, 41, 43, 48, 49, 50, 51, 52, 60, 121, 140, 141). The objective of such isolation schemes generally has been to precipitate the whey proteins in an undenatured form, followed by partial or complete removal of the coagulating agent. Few attempts have been to characterize functionality of the protein-polymer complex itself. However, depending upon the specific polymer used, the complex might possess certain interesting and useful attributes.

Complexing between proteins and acidic polysaccharides often results in a more stable protein conformation, reflected by greater resistance to heat denaturation (6, 127). Hidalgo and Hansen (48), while investigating the interaction between β-lactoglobulin and several food stabilizers, noted that CMC afforded some thermal stability to the protein at neutral pH (Figure 5). Measurement of exposed sulfhydryl groups revealed that enhancement in heat stability was not derived from altered protein conformation. Instead, thermal protection may arise from a physical barrier (i.e., CMC) that prevents protein-protein interaction and aggregation. A similar view of biopolymer thermal protection has been stated by Tolstoguzov et al. (127).

Hansen and Black (41) evaluated a spray-dried complex of whey protein with CMC as a potential substitute for egg white in meringues. When adjusted to the pH of egg white, pH 8.5, the complex produced whipped foams comparable to egg white foams in appearance and texture, as well as in specific volume and stability. Mann et al. (79) successfully incorporated a spray-dried complex of whey protein with CMC into cake mix as a total replacement for egg. In a study of the functional properties of whey protein concentrates isolated by six techniques, Morr et al. (92) observed that the complex of whey protein with CMC

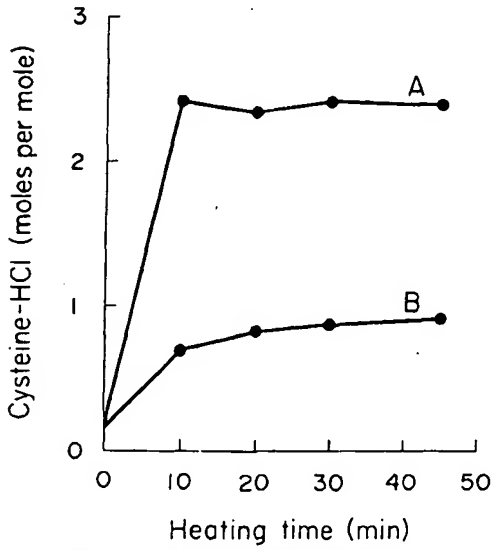


Figure 5. Effect of carboxymethyl cellulose (CMC) on heat-induced release of sulfhydryl groups of β-lactoglobulin at neutral pH (48) (.6% protein, .2% CMC, 80°C, ionic str. <.02). A) Control; B) with added CMC. Reprinted from *Journal of Agricultural and Food Chemistry*. Copyright 1969, American Chemical Society.

had almost twice the oil emulsifying capacity of any of the other whey protein preparations. In addition, the CMC complex produced the most stable foam upon whipping.

Although there have been few reports on functional behavior of complexes of whey protein with biopolymer, the limited results do suggest that this is an approach for protein modification that should be explored in greater detail. When incorporated into an efficient protein isolation scheme, complexing with biopolymers may prove to be a viable means of whey protein modification both economically and aesthetically.

The ability of proteins to be texturized is a functional property of increasing importance in food product development (66). Protein texturization can broaden the range of food applications to include use as meat analogues or extenders. Textured proteins must possess sufficient structural integrity to withstand high temperature processing and cooking. The techniques most often used to impart the necessary structure are fiber spinning and thermoplastic extrusion. Fiber spinning has been applied successfully to preparation of continuous fibers from whey protein concentrate (5, 55). Fibers of adequate strength were formed when a detergent (sodium dodecyl benzene sulfonate) was added to the alkaline spinning solution. Fibers were prepared by extrusion of the spinning solution containing whey protein and detergent into an acetic acid-sodium chloride coagulating bath. Residual detergent was extracted with acetone, yielding whey protein fibers with excellent handling properties, bland flavor, and acceptable mouthfeel.

CONCLUSIONS

If modification is to become a common practice to improve or extend functional behavior of whey proteins, a number of unanswered questions will have to be investigated in great detail. Perhaps most important are the effects of modification upon amino acid bioavailability and the toxicologic consequences of certain chemical derivatizations. Chemical modifications that involve reaction with the ϵ amino group of lysine may be detrimental to availability of this essential amino acid. In this regard, amidation or esterification of acidic amino acids in proteins may be more acceptable. However, when considering the nutritional impact of modifying amino acid side chains, the practical relevance of amino acids that are rendered unavailable should be weighed. Proteins that are modified chemically to alter functionality probably will not be relied upon as an essential dietary source of nutritive protein. Even if the protein in question does constitute a significant portion of protein intake, complementary feeding may make the lowered availability unimportant.

Of greatest concern with respect to chemical modification is the potential for harmful by-product formation. In addition, residual traces of the modifying reagent that may remain after completion of the derivatization present toxicological problems. Thorough testing to evaluate critically safety and acceptability will be required prior to adoption of any chemical techniques to alter protein functionality. This does not imply, however, that research directed toward altering protein function via chemical means is of little value. On the contrary, such investigations provide a great deal of useful information concerning the relation between protein conformation and functional behavior. A basic understanding of the role that structure has in determining functionality will be required before modification schemes can be designed intentionally to tailor properties of whey proteins to fill specific food applications. Furthermore, investigation of effects chemical modification has on protein conformation and function may reveal less drastic methods for improvement of functionality, such as enzymatic derivatization and complexing with various food grade polymers.

As development of fabricated food continues to increase in scope, the requirement for food proteins with well-defined functional performance will become of paramount importance. This presents a unique opportunity to the dairy industry, as there is a readily available and abundant supply of surplus protein. The challenge is to develop safe and inexpensive methods for modifying whey proteins to extend or alter the combination of functional properties. Such intentional modification ultimately will make whey proteins useful additives in formulation of a wide variety of food products.

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Functionality of Extrusion—Texturized Whey Proteins[1]

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ABSTRACT

Whey, a byproduct of the cheesemaking process, is concentrated by processors to make whey protein concentrates (WPC) and isolates (WPI). Only 50% of whey proteins are used in foods. In order to increase their usage, texturizing WPC, WPI, and whey albumin is proposed to create ingredients with new functionality. Extrusion processing texturizes globular proteins by shearing and stretching them into aligned or entangled fibrous bundles. In this study, WPC, WPI, and whey albumin were extruded in a twin screw extruder at approximately 38% moisture content (15.2 ml/min, feed rate 25 g/min) and, at different extrusion cook temperatures, at the same temperature for the last four zones before the die (35, 50, 75, and 100°C, respectively). Protein solubility, gelation, foaming, and digestibility were determined in extrudates. Degree of extrusion-induced insolubility (denaturation) or texturization, determined by lack of solubility at pH 7 for WPI, increased from 30 to 60, 85, and 95% for the four temperature conditions 35, 50, 75, and 100°C, respectively. Gel strength of extruded isolates increased initially 115% (35°C) and 145% (50°C), but gel strength was lost at 75 and 100°C. Denaturation at these melt temperatures had minimal effect on foaming and digestibility. Varying extrusion cook temperature allowed a new controlled rate of denaturation, indicating that a texturized ingredient with a predetermined functionality based on degree of denaturation can be created.

(**Key words:** extrusion, texturization, whey proteins, functionality)

Abbreviation key: WLAC = whey lactalbumin, **WPI** = whey protein isolate.

INTRODUCTION

Though new products incorporating whey proteins, such as sports drinks, are being developed, innovation in process and product development is still needed (American Dairy Products Institute, 2000). Fortifying snacks with whey proteins could provide a particularly attractive outlet for surplus whey proteins. This practice has been limited, however, due to known adverse textural effects when the whey protein concentrate supplementation is greater than 10% of the main starch component (Kim and Maga, 1987). It has been the hope that successful incorporation of whey into extruded products will increase use of whey products and improve the nutrient density of snacks by increasing the protein content. However, using nontexturized whey proteins has not been successful in improving functional qualities of coextruded puffed products, especially their expansion and crunchiness (Singh et al., 1991).

Adding proteins to extruded starch-based snacks increases the number of sites for crosslinking, but shorts the starch matrix, resulting in tough, nonexpanded crusts (Martinez-Serna and Villota, 1992). The source, concentration, and processing conditions of the starting whey proteins are significant factors, since partial or complete denaturation affects their structure and leads to changes in crosslinking and complexing of the whey proteins during extrusion (Aboagye and Stanley, 1987). Proteins can be pretexturized or denatured before adding to the extruder to create a better matrix, which could lead to improved functionality (Kester and Richardson, 1983; Mohammed et al., 2000).

Extruders, with their shearing screws operating at varying speeds, impart significant structural changes to food components including proteins (Harper, 1981). A demonstrated benefit of high shear processes is the ability to change the molecular structure of proteins (Kollengode et al., 1996; Batterman-Azcona and Hamaker, 1998). Though protein denaturation by thermal extrusion is not well established (Taylor and Fryer, 1994), whey proteins can be modified using chemicals, heat, or by shear in the extruder (Kim and Maga, 1987). Chemical treatment alone alters the reactive groups of the amino acids, resulting in changes in the noncovalent forces that influence conformation such as van der Waals forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonding (Kester and Richardson, 1984). Heating and shear alter the conformational

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structure of the protein through partial denaturation of the protein, thereby exposing groups that are normally concealed in the native protein (Kim and Maga, 1987).

To increase utilization of whey proteins, effort is needed to improve their functionality in the presence of other components such as starches, flours, and other nondairy proteins. This can be accomplished by first texturizing the whey proteins through denaturing their globular structure, or unfolding and realigning the proteins, before inclusion with other matrices. Therefore, our objectives in this work were to texturize and modify the functionality of whey proteins to create new uses for them.

MATERIALS AND METHODS

Whey protein concentrate (ALACEN 834) and lactalbumin (ALATAL 825) were purchased from New Zealand Milk Products, Inc. (Santa Rosa, CA). Whey protein isolate (**WPI**; PROVON 190) was purchased from Glanbia Ingredients. The compositions were as follows: whey protein concentrate: moisture 2.8%, protein 83.6%, fat 0.8, ash 3.3%, and carbohydrate by difference; whey lactalbumin (**WLAC**): moisture 5.5%, protein 89.9%, fat 3.8, ash 0.5%, and carbohydrate by difference; WPI: moisture 2.8%, protein 89.6%, fat 25, ash 3.3%, and carbohydrate by difference.

A ZSK-30 twin screw extruder (Krupp Werner Pfleiderer Co., Ramsey, NJ) with a smooth barrel was used. The extruder has nine zones, and the effective cooking zones 6, 7, 8, and 9 were set to the same temperature for each test. To achieve different melt temperatures the cooking zones were set to the same barrel temperature 35, 50, 75, or 100°C, respectively. Zones 1 to 3 were set to 35°C and zones 4 and 5 were set to 75°C. For example, the profile for extrusion cook temperature 35 was: 35, 35, 35, 75, 75, 35, 35, 35, 35. Melt temperature was monitored behind the die. The die plate was fitted with two circular inserts of 3.18 mm diameter each. The screw elements were selected to provide low shear at 300 rpm; the screw profile was published by Onwulata et al. (1998). Feed was conveyed into the extruder with a series 6300 digital feeder, type T-35 twin screw volumetric feeder (K-tron Corp., Pitman, NJ). The feed screw speed was set at 600 rpm, corresponding to a rate of 3.50 kg/h. Water was added into the extruder at the rate of 1.0 L/h with an electromagnetic dosing pump (Milton Roy, Acton, MA). Samples were collected after 25 min of processing, freeze-dried overnight in a VirTis Freeze Mobile 12XL Research Scale Freeze Dryer (Gardiner, New York), and stored at 4.4°C until analyzed. The experiments were performed in triplicate.

Analysis of variance was used to identify differences in physical properties at various processing conditions. Duncan's multiple range test was used for mean separation, and correlation coefficients were calculated. The Statistical Analysis System (SAS) package was used (SAS Institute Inc., Cary, NC) in all cases. Significance was defined as $P = 0.05$.

Moisture was determined by the AOAC Official Method 925.10. Extrudate samples weighing approximately 1.5 g were dried in a vacuum oven at 100°C overnight (AOAC, 2000).

Ash was determined by the AOAC Official Method 923.03. Ash was determined for each sample using 3 g assayed in a Muffler furnace at 550°C for 16 h; percentage of ash was calculated.

Fat was determined using the AOAC Official Method 30-25. One gram of extrudate was placed in an Erlenmeyer flask and 1 ml of sulfuric acid and 4 ml water was added to the flask. The samples were mixed gently and after 60 min were transferred to a 60-ml separatory funnel using 25 ml of dichloromethane:methanol solution (1:1). Extrudate sample were shaken and allowed to separate for 15 min. The bottom layer was drained into a weighing pan and then evaporated, and the amount of fat was determined (AACC, 1995).

Protein was determined with 0.2 g of extrudate analyzed with the LECO Protein Analyzer Model FP2000 (LECO Corporation, St. Joseph, MI). Percent protein was calculated with the N conversion factor 6.38 for whey protein.

Gel strength was measured by Bloom determinations with a TA-XT2 Texture Analyzer as described by Ju and Kilara (1998). A 12% WPI solution was made (3.204 g of ground freeze-dried sample mixed with 26.7 ml of deionized water and 3.3 ml of 0.03 M $CaCl_2$), and allowed to sit for 15 min in a 50- × 70-mm cylindrical jar. The sample was heated to 80°C for 30 min in a water bath, cooled in an ice bath for 15 min, and then stored overnight at 4°C. The specimen was thawed at 25°C in a 50% relative humidity room. Gel strength was determined with a TA-XT2 Texture Analyzer running a penetration test with a 30-mm analytical probe to a depth of 6 mm at the rate of 1 mm/s. The weak gels were easily deformed with evidence of syneresis.

Protein insolubility was determined with 1.0 g of ground freeze-dried extrudate sample mixed with 90 ml of deionized water. The protein suspension was stirred at 125 rpm, at pH 7.0 for 2 h. The suspension was centrifuged for 20 min, and the supernatant was freeze-dried overnight. The LECO Protein Analyzer Model FP2000. was used to analyze the solids from the supernatant for protein content. Protein insolubility (denaturation) was calculated as described by Kilara

(1984) as: [% total protein – % soluble protein = % insoluble (denatured)].

Foam volume and stability of extruded proteins were determined by heating 2.3-g samples mixed with 35 ml of deionized water to 60°C for 15 min. The slurry was then whipped for 15 s in Waring Lab Micronizer FPC70 (Waring Products Division, New Hartford, CT), then transferred to a 100-ml graduated cylinder where the foam volume was read initially, and then every 5 min for 1 h. Foam stability (foam capacity at specific time) over the 1-h period was calculated.

Protein digestibility was determined with 10 ml of extrudate sample dissolved in distilled water. The pH was then adjusted to 8.0 with 0.1 *N* NaOH or HCl. One milliliter of freshly prepared enzyme stock solution (1.6 mg/ml trypsin, 3.1 mg/ml Chymotrypsin, and 1.3 mg/ml aminopeptidase) was added to the protein suspension at 37°C. The pH after 10 min was recorded with a portable pH meter (IQ Scientific Instruments, Inc., San Diego, CA), and a Tris/HCl buffer containing 2.0% SDS (wt/vol) and 0.1% mercaptoethanol (vol/vol) was added to the protein solution which was immediately heated to 90°C to determine the enzymatic reaction. Samples were then analyzed by quantitative gel electrophoresis. The percent protein digestibility was calculated by the following equation: %digestibility = 210.46 B 18.10(X), where X is the pH (Ju and Kilara, 1998).

For SDS-PAGE assay, samples were vortexed and dissolved in 20 m*M* Tris/HCl, 5 m*M* EDTA, 2.5% SDS with and without 5.0% 2-mercaptoethanol at pH = 8.0 then heated in boiling water for 2 min. Bromophenol blue was added to about 0.1% concentration. The samples were at 2 mg/ml concentration. Phast gels (Amersham Pharmacia Biotech, Uppsala, Sweden) were run according to the procedures given by the manufacturer for SDS 20% homogeneous gels. The 6-lane (4 μl per lane) sample applicators were used. Protein staining used the Coomassie blue procedure given by the manufacturer (Farrell et al., 1998).

For fine structure, transmission electron microscopy was done of thin sections made from epoxy-embedded samples. Millimeter-sized pieces of coarsely ground, freeze-dried segments of ribbons of the extrudates were immersed in 2.5% glutaraldehyde in 0.1 *M* imidazole buffer solution (pH 6.8) and stored in sealed vials at 4°C. For embedding and thin sectioning, the segments were washed in imidazole buffer, immersed in 2% osmium tetroxide in 0.1 *M* imidazole buffer for 2 h at room temperature, washed in distilled water, and gradually dehydrated in a series of ethanol solutions and propylene oxide for 1 h. Samples were then infiltrated with a 1:1 mixture of propylene oxide and epoxy resin mixture overnight and finally embedded in epoxy resin. Thin sections were cut and stained with 2% uranyl acetate, and lead citrate solutions. The transmission electron microscopy was done in the bright field mode using a model CM12 electron microscope (FEI/Philips, Hillsboro, OR). Average spacings of electron density, corresponding to fine structure in the extrudates, were estimated from the intensity distribution in Fourier transforms computed from digital images made from transmission electron microscopy photographic negatives recorded at 45,000×. Negatives were digitized using a SprintScan 45 film scanner (Polaroid Corp., Cambridge, MA) and square areas of 2.8 MB images (512 × 512 pixels) were transformed after flattening, adjustment of brightness and contrast and one cycle of a low pass filter using a 3 × 3 pixel kernel in Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Line profiles of the radial distribution of intensity in the Fourier transforms were made, and reciprocal spacings were calculated based on the location of orders of peaks in transforms of a line grating with an equivalent spacing of 22 nm.

For scanning electron microscopy, a layer of dry powder particles was adsorbed onto conductive carbon adhesive tabs glued to aluminum specimen stubs (Electron Microscopy Sciences, Ft. Washington, PA), and the surface was coated with a thin layer of gold in a model Scancoat Six sputter coater (BOC Edwards, Wilmington, MA). Images of the powder particles were made with a model JSM 840A scanning electron microscope (JEOL USA, Peabody, MA) operating in the secondary electron imaging mode and integrated with a digital image workstation, model Imix1 (Princeton Gamma-Tech, Princeton, NJ).

Table 1. Extrusion melt temperatures of whey proteins.

Product	Melt temperature (EC)	Preextrusion (%)	Postextrusion (%)
WPC80[1]	70[b]	40.9[b]	59.9[b]
WLAC[2]	75[a]	68.7[a]	94.4[a]
WPI[3]	74[a]	28.0[a]	94.8[a]

[a,b,c]Means with different letters within a column are significantly ($P < 0.05$) different.

[1]WPC80 = Whey protein concentrate, 80% protein.

[2]WLAC = Whey lactalbumin.

[3]WPI = Whey protein isolate: number reported is mean of three samples. The preextrusion and postextrusion numbers are percent insoluble protein.

RESULTS AND DISCUSSION

Extruding whey proteins at the cook temperature of 75°C resulted in varying degrees of melt temperatures and denaturation for the different products (Table 1). Following extrusion, whey protein concentrate was the least denatured and WLAC and WPI were significantly

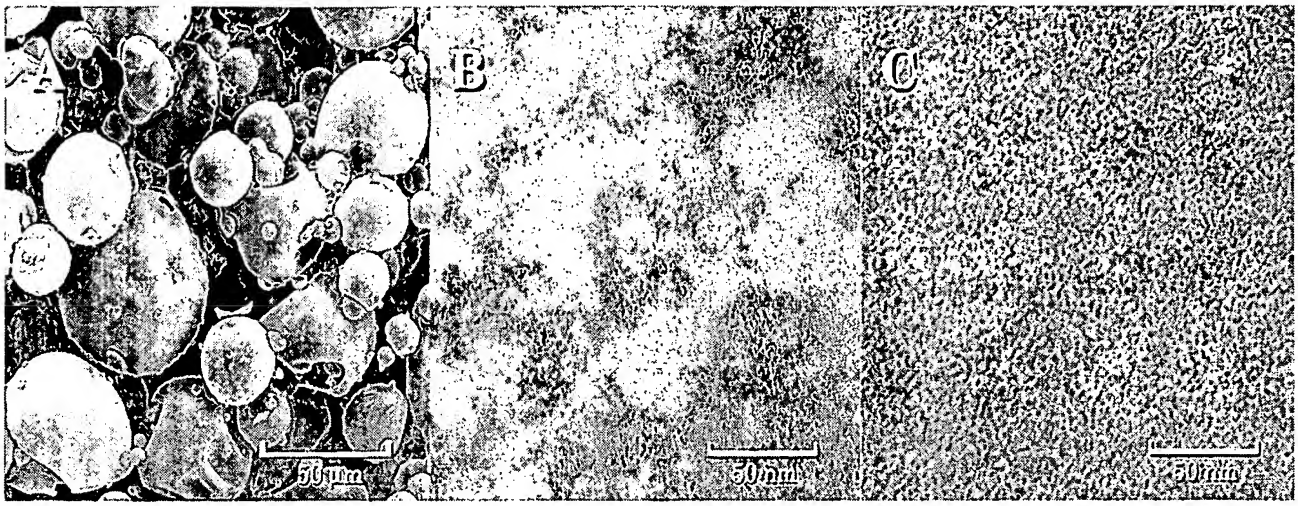

Figure 1. Electron micrograms of whey protein isolates (WPI). (A) Scanning microscopy was used to examine dry powder. (B) The nonextruded WPI paste (40% moisture) was embedded, stained with uranyl acetate and sections examined by transmission electron microscopy; (C) Extruded (100°C) WPI (40% moisture) treated as in (B).

($P < 0.05$) more denatured. Although WPI and WLAC were equally denatured, because of the wider spread in insolubility between the initial and final WPI values, from 28 to 94.8%, further experiments were conducted with WPI.

The effect of extrusion cooking on denatured proteins was examined by electron microscopy. Changes in the microstructure of WPI and the ultrastructure of the denatured proteins are presented in Figure 1. The microstructure of the dry powders, examined by scanning electron microscopy, reveal particles ranging from 10 to 50 μm in diameter (A). Transmission electron microscopy (B) shows the release of protein at the edge of powder particles after brief exposure to water typical of initial mixing in the extruder. Here irregular strings and granules, corresponding to molecular aggregates, ranging from less than 10 nm to over 200 nm can be seen (B). In contrast, the ultrastructure of extruder-denatured insoluble whey protein shows a closely packed arrangement of electron dense particles, typical of denatured protein matrix, ranging from approximately 2 to 6 nm in diameter (C).

As the whey proteins denature, they become insoluble and aggregate (Walstra et al., 1999). Changes in globular protein structure occur at or above 50 and 80°C for α-lactalbumin and β-lactoglobulin, respectively (Farrell et al., 2002; Hong and Creamer, 2002). However, with the addition of shear in the extruder, significant unfolding (denaturation) occurred at 50°C. Other conditions that favor denaturation are pH conditions lower 4.6 for whey proteins (Harwalker, 1979). WPI extruded at cook temperature 50°C or above, denatured significantly ($P < 0.05$) with increased cook temperature, increasing in order of magnitude every 25°C. The pH of the suspended protein remained stable as extrusion temperature increased, but measurable N (protein) increased as shown in Table 2. The heat denaturation temperature for whey in solution ranges from 50 to 90°C for 30 min (McClements and Keogh, 1995; Ennis and Mulvihill, 2000). Thermal denaturation takes place at 50 and 75°C unfolding and unmasking the SH groups (Linden and Lorient, 1999). The level of denaturation and subsequent insolubility at pH 7 depends on heating temperature and time, and the pH of whey at heating (Ennis and Mulvihill, 2000). Whey in solution is heated up to 30 min and lost protein N as temperatures increased above 80°C, but we observed no significant change in protein N content after drying. Though the amount of protein denatured increased, with increasing

Table 2. Properties of WPI as function of extrusion temperature.

Extrusion cook temperature	Melt (°C)[1]	pH	Protein[2] (%)	Insoluble (%)	Digestibility (%)
35	39^{d}	6.7^{b}	90.7	28.4^{c}	89.6^{a}
50	48^{c}	6.8^{ab}	90.9	3.3^{c}	88.2^{ab}
75	66^{b}	6.9^{a}	91.7	77.7^{b}	85.7^{bc}
100	92^{a}	7.0^{a}	91.4	87.2^{a}	84.5^{c}

[a,b,c]Means with different letters within a column are significantly ($P < 0.05$) different.

[1]Extrusion melt temperature at the die.

[2]Protein percentage after drying. Properties of nonextruded WPI: pH 6.8, protein 88.9%, insoluble (denatured) 28.0%, and digestibility 87.7%.

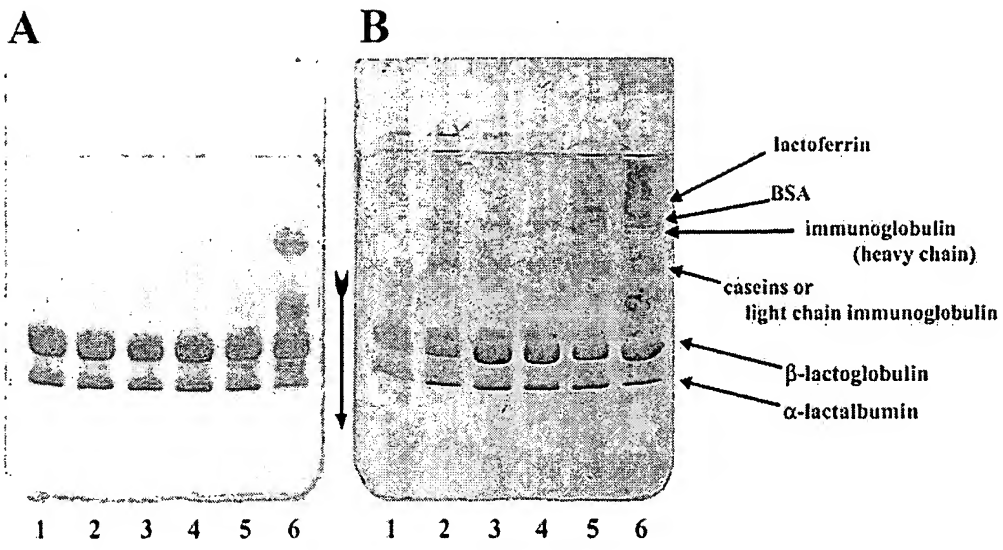

Figure 2. SDS-PAGE of extruded whey isolates. (A) With 2-mercaptoethanol; (B) without 2-mercaptoethanol. The lanes are: 1 = 100°C; 2 = 75°C; 3 = 50°C; 4 = 35°C; 5 = Native WPI; 6 = laboratory whey.

temperature, denaturation had minimal overall effect on protein digestibility. So, the interesting result is increased protein denaturation without a significant loss of digestibility due to extrusion below 100°C. Extrusion denaturation occurs in the short time order of 45 to 90 s within the extruder. The short time might explain why extrusion texturized WPI maintains its digestibility.

The WPI and variously heat-treated samples were compared by SDS-PAGE (Figure 2). The SDS gel of the variously denatured WPI, indicated minimal change in solubility (Figure 2). The SDS gels were initially developed without reducing reagent so the protein disulfide bonds are intact. The unreduced samples at 35 and 50°C show somewhat diminished bands for the higher molecular weight whey proteins (B). However, at 50 and 75°C samples were equivalent weight, and fainter than the native whey or whey proteins produced in the laboratory on the SDS gel (compare lanes 1 and 2 with 6 in Figure 2). In this respect, the SDS gels parallel the solubility data in that increased temperature decreases solubility in SDS alone, indicating sulfhydryl-disulfide crosslinking. When the samples were reduced thoroughly and all disulfide bonds cleaved, all the extruded whey samples at the different temperatures were similar to each other and to the initial WPI (A). Thus, extruding whey even at the highest temperatures does not affect the overall protein ratios. The native and extruded whey still have the same amount of the different proteins (Figure 2) and their total N values were similar (Table 2). Soy proteins and gluten are two systems that are generally extruded at high temperature and low moisture contents to form structured products. Their solubility is high, thus requiring that their hard-to-break disulfide bonds be dissolved with high solubility solvents such as β-mercaptoethanol and sodium dodecyl sulfate (SDS). Our denatured whey protein system behaved similarly to soy protein and gluten, showing a similar pattern of bonding and crosslinking. Whey isolates were most denatured by heat of different proteins extruded (Mohammed et al., 2000).

Physical functional properties of extruded WPI such as gel strength, foam volume, and stability were significantly affected at and above 75°C and were proportionally affected at lower preset temperatures. Greater than 30% moisture was needed to extrude the whey protein isolates, but the only significant change in mois-

Table 3. Physical properties whey protein isolate (WPI) as function of extrusion temperature.[1]

Extrusion cook temperature	Moisture (%)	Gel strength (N)	Foam volume (%)	Foam stability
35	42.5[a]	114.9[b]	298.1[ab]	29.8[ab]
50	40.9[b]	145.3[a]	301.9[a]	30.2[a]
75	42.6[a]	2.8[c]	173.3[b]	17.3[c]
100	38.9[c]	—[2]	77.1[c]	7.7[c]

[a,b,c]Means with different letters within a column are significantly ($P < 0.05$) different.

[1]Properties of nonextruded WPI: moisture 1.94%, gel strength 52.3 (N), foam volume 288%, and foam stability 28.7%.

[2]Value not reported.

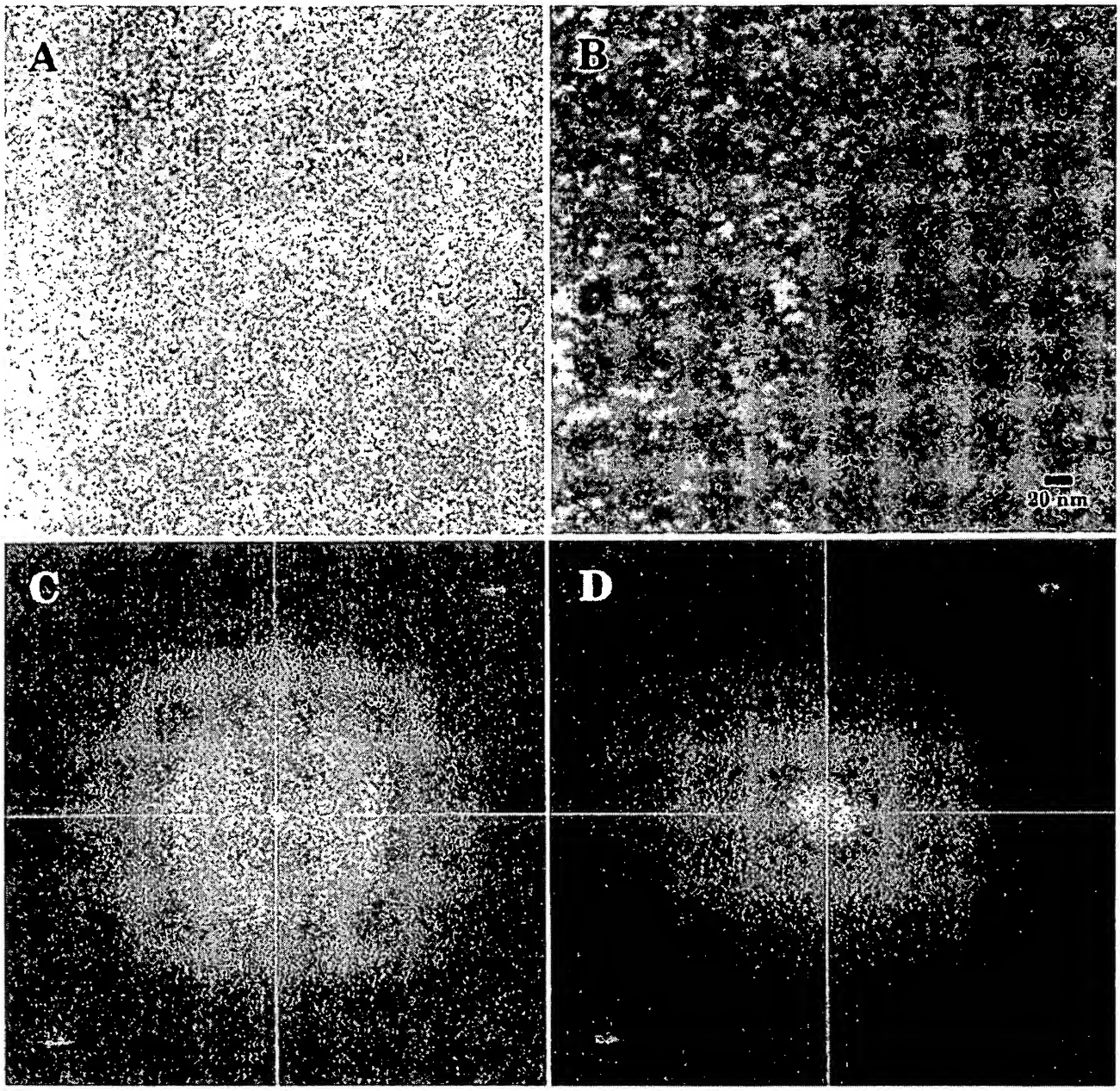

Figure 3. Transmission electron micrographs of whey protein isolates (WPI) positively stained with uranyl acetate and lead citrate; (A) Enlargement of denatured whey extruded at 100°C, as in Figure 1C; (B) enlargement of a selected protein-dense area of Figure 1B, undenatured paste of WPI; (C) fast Fourier transforms of electron density images, native and denatured (D).

ture of the extruded products occurred at 100°C (Table 3). Partial denaturation at temperatures between 35 and 50°C significantly increased gel strength, but at 75°C or higher complete loss of gelling property resulted. Foam volume remained high up to 50°C but decreased significantly ($P < 0.05$) after 75°C. Foam stability followed the same pattern as volume, being very stable for an hour below 50°C. This result is contrary to the report that WPI, heated to 80°C had little effect on stability (Phillips et al., 1990). However, with the addition of shear from the extruder, we observed significant loss of volume and stability.

Denatured whey protein isolate looks quite different from the nondenatured proteins at the ultrastructural

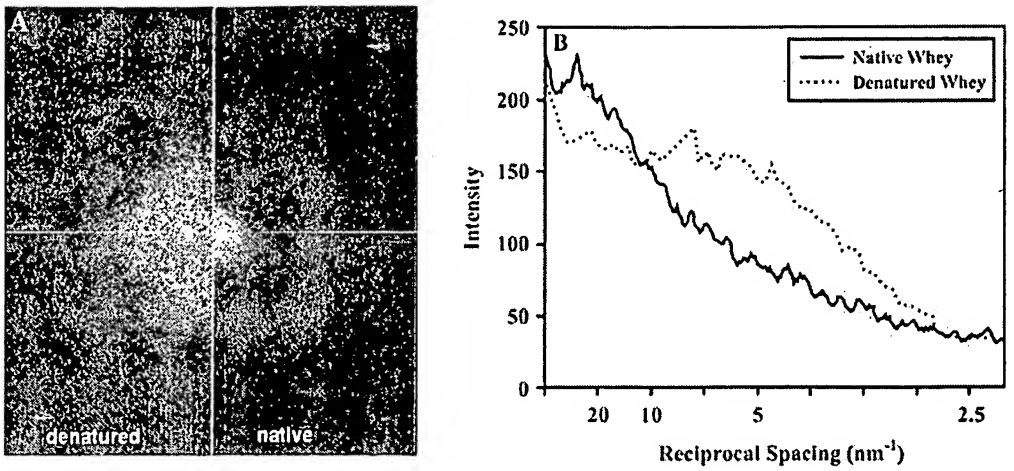

Figure 4. Electron-density mapping corresponding to the Fourier transforms (A) for denatured (extruded at 100°C) and native whey protein isolate (WPI), and (B) inverse reciprocal spacing of electron-density images of native and denatured WPI.

level (Figure 3). As sampled, denatured proteins (3A) (WPI extruded at 100°C) are densely packed with spacing of 2 to 6 nm, while nondenatured whey in the paste are loosely packed with a large spacing 200 to 350 nm (3B). The differences in fine structure of denatured and native whey protein are illustrated in Figures 3 and 4. In the "native" whey protein (40% slurry), the distribution of electron density surrounding the hydrating particles (Figure 1B) is an open network with clear, electron-lucent spaces ranging from 15 to 40 nm and irregular structures of electron density of similar dimensions. In contrast, the fine structure in segments where the whey proteins are completely denatured is limited to close-packed fine granules around 3 to 8 nm in diameter (Figure 3). The corresponding computed Fourier transforms indicate that images of extrudate containing native whey proteins consist mainly of low spatial frequencies, indicating structures with average spacings ranging from 15 to over 40 nm, whereas images of extrudate containing denatured whey proteins have little intensity at low spatial frequencies but high intensity corresponding to high spatial frequencies, relating to electron density changes ranging from about 3 nm to less than 10 nm (Figure 4). The constraint of extruding whey is keeping the temperature below the point where pyrosis will occur, as evidenced by relatively constant N content (Table 2). Texturized whey products are sometimes extruded at 150°C to form meat-like stringy structures (Lin et al., 2000). It is thus possible that whey proteins extruded at a temperature higher than 100°C would form very dense fibrous structures. We have seen evidence of fine structures with transmission electron microscopy images at 100°C in whey isolates. There is further evidence that texturized whey products could function as food adjuncts. In a consumer taste, extrusion texturized whey protein concentrate (80% protein) was shown to be comparable to texturized soy protein (Hale et al., 2002).

We have created structured networks in whey proteins using mild heat and shear to create reversible texturized whey proteins. By understanding the effects of shear on a molecular basis, ways of creating new functionality can be developed. This will enable development of extrusion parameters that permit controlled denaturation of whey proteins.

CONCLUSION

Extrusion processing denatured whey protein concentrates, WLAC and WPI, but the greatest amount of denaturing occurred with WPI. Denatured whey protein isolate retained its native protein value, functionality, and digestibility when extruded at 50°C or below; changes in functionality occurred at 75 and 100°C. Through careful selection of extrusion conditions, denatured whey proteins with unique functionality were produced. Texturization increased with temperature, but temperatures higher than 100°C may be needed to form texturized fibrous products from whey protein isolates. We show here that extrusion is an effective tool for denaturing whey proteins to create texturized products. Thermally denatured WPI is a unique ingredient that can be used in large amounts in nontraditional applications such as in acidified shelf-stable beverages.

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Cheese Whey Proteins Isolated with Polyacrylic Acid

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ABSTRACT

Cottage cheese whey and sweet whey proteins have been recovered from fresh whey solutions by reversible precipitation with polyacrylic acid. The work describes the method of protein isolation and characterizes the final product by electrophoresis, proximate analysis, mineral analysis, essential amino acids, and solubility in buffers. A unique functional property of the isolated cheese whey protein preparation is coagulation by heating into a firm egg-white-like gel. The water holding capacity, textural properties, coagulation temperature, and least coagulable protein concentration are compared to commercial egg white. Replacement of egg white in a cake gave equal acceptability by a test panel.

INTRODUCTION

Interest in recovery of cheese whey proteins is emphasized by the large number of publications concerning the subject (15). Fresh cheese whey contains an average of .5% protein of very good nutritional value with a variety of functional properties useful in food applications. Functional properties of whey proteins important for food applications are largely dependent on the particular method of isolation. Heat, pH extremes, chemical precipitants, or mechanical shear cause degrees of denaturation with consequent loss of functional properties in the native proteins. The loss of functional properties decreases the number of possible applications, especially those based on solubility and thermal coagulation. Competition from other nutritional protein ingredients such as milk solids, casein, and egg white connects the market value of a commercial whey protein to the diversity of applications rather than to its intrinsic nutritional quality. In a study of whey protein samples prepared by seven separation methods, Morr et al. (15) revealed differences of composition, initial pH of solutions, solubility, emulsification, whipping properties, and buffer capacity. Apparent denaturation of the protein was 22% for an electrodialysis isolated product and 90% for whey protein obtained by metaphosphate precipitation.

Chemical precipitation methods have been favored by a number of authors. Amantea et al. (1) reviewed precipitation with ferric salts and described the preparation and properties of a soluble ferric whey protein. Richert et al. (19) reported on precipitation with hexametaphosphate. Gordon (6) was granted a patent for separating proteins with metaphosphates. Jones et al. (11) used Ferripolyphosphate, and Murray (16) used tannic acid. Hansen et al. (8) precipitated whey proteins with carboxymethylcellulose, while Hidalgo and Hansen (9) in a study of the interaction of β-lactoglobulin with food stabilizers reported complexing with anionic polysaccharides.

Sternberg and Hershberger (23) found that proteins can be precipitated by polyacrylic acids and recovered essentially undenatured and purified. The method offers operational simplicity, at comparative low cost using small concentrations of an inexpensive reagent. In this paper, we report the recovery of cheese whey proteins with polyacrylic acid and characterization of the isolated product.

MATERIALS AND METHODS

Fresh cottage cheese whey was obtained weekly from Burger Creamery, New Paris, IN. Storage between deliveries was at 4 C. Sweet whey was obtained from County Line Cheese Company in Auburn, IN. Polyacrylic acid of approximate molecular weight 90,000 was a commercial material Good-Rite K 702 from B. F. Goodrich Company, described in their bulletin GC-51. Dried egg white solids were from Henningsen Foods Inc. Whey protein recovered by ultrafiltration was SOLAC 500 from New Zealand Milk Products described in the manufacturer's technical service bulletin SOLAC.

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Functional properties were determined on cottage cheese whey protein isolate dried by spray drying in a Nichols/Niro utility spray dryer Model II at 140 to 150 C input temperature.

Protein Recovery

A sample of 500 ml of cottage or cheddar cheese whey at a temperature of 18 C was adjusted to pH 4.0 with 2.5 M H_3PO_4, mixed with 1.5 g filter aid and 3 ml of a 25% polyacrylic acid solution. After 10-min stirring, the precipitate was allowed to settle for 1 h and the supernatant siphoned off. Solids were separated by vacuum filtration, suspended in 100 ml water, and mixed for 1 h with .6 g of magnesium carbonate which increased the pH to 6.5. After filtration, the solution containing the protein was concentrated in vacuum at 40 C up to 20% total solids and freeze-dried. The cake containing filter aid and magnesium polyacrylate was suspended in a volume of water equal to the initial volume of polyacrylic acid, adjusted with 5 N H_2SO_4 to pH 1.5 and recycled into a fresh batch of cheese whey.

Amino Acid Analysis

Samples of cottage cheese whey and sweet whey proteins isolated with polyacrylic acid were hydrolyzed for 4 h at 120 C with 6 N HCl in sealed ampules. The amino acids released in the hydrolysates were determined with a Technicon ion exchange analyzer and expressed in grams of amino acid per 100 g protein. Tryptophan was determined by the p-dimethylaminobenzaldehyde color reaction (21).

Analytical Methods

The AOAC procedures (2) were followed for fat by the Mojonnier extraction, ash, chlorine, and micro-Kjeldahl nitrogen. The factor for converting total nitrogen values into protein was 6.38. Nonprotein nitrogen was obtained after precipitation of proteins in 12% trichloroacetic acid. Lactose was determined with phenol sulfuric acid (12), phosphate by the ammonium phosphomolybdate colorimetric method of Fiske and Subbarow (5), and lactate as lactic acid by liquid chromatography (17) with a Waters Associates instrument ALC-100. Elemental analyses were obtained by atomic absorption with a Perkin-Elmer instrument Model 403 on a 1% whey protein solution adjusted to pH 5.0 with 1 N HCl.

Electrophoresis

Samples of whey protein were analyzed with polyacrylamide disc gel electrophoresis (3) using as running gel buffer 377 mM TRIS(2-amino-2-hydroxymethyl-1,3-propanediol) titrated to pH 8.9 with HCl, and 38 mM glycine titrated with TRIS to pH 8.2 as tank buffer solution. The actual time of electrophoresis was 70 min at 1 to 3 mA/sample. After staining with aniline blue-black, the individual fractions were traced with a Photovolt Densicord Densitometer (Photovolt Corp., New York, NY) and expressed as percent of total traced area.

Solubility Determinations

Solutions of 1% protein were made in distilled water then adjusted to pH 2 to 9 with 1 N HCl or 1 N NaOH. After mixing for 30 min at 22 C, all solutions were centrifuged at 1000 x *g* for 10 min. Total N was determined in supernatants and expressed in percent of the value of N in the sample before solubilization. Solubility at pH 6.5 was determined by dissolving various amounts of protein in distilled water. After mixing for 30 min, the samples were centrifuged, nitrogen was determined in supernatants and related to the amount of nitrogen used to prepare the solution.

Least Coagulable Protein Concentration

Solutions of whey protein isolate and egg white were made in .1 M sodium acetate-acetic acid buffers for the pH range 4.5 to 5.5 and in .1 M sodium phosphate buffer for the range of pH 6.0 to 8.0. A volume of 2 ml from each solution was dispensed in 150 x 16 mm test tubes. The tubes were immersed in a boiling water bath for 30 min, then cooled for 5 min in tap water. Coagulation was judged complete (10) if the coagulum was not fluid when the tubes were inverted.

Coagulation temperature. Coagulation temperature was determined on a 16% wt/vol protein solution in distilled water at pH 7.0. The temperature was varied 60 C to 75 C with 2 C increments. Samples of 2 ml in 150 x 16 mm test tubes were kept 30 min at the respective temperature, cooled 5 min in tap water, then inverted to judge coagulation.

Relationship Between Coagulation Time and Concentration of Lipids in the Protein Solution

Portions of 5 g cottage cheese whey protein were extracted 4 h in a Soxhlet extractor with 150 ml chloroform-methanol mixture (2:1 vol/vol). The extract was evaporated in vacuum at 20 C, and various amounts of lipids from the residue were emulsified by rapid mixing with a water solution of 10% wt/wt of cottage cheese protein isolate adjusted to pH 7.0 with .5 N NaOH. The coagulation time at 68 C was determined by heating 2 ml solutions in 150 x 16 mm test tubes and measuring the time elapsed until the solution did not run out when the test tube was inverted.

Water Holding Capacity

Two milliliter aliquots of 10% protein solutions adjusted to pH 6.0, 7.0, 8.0, and 9.0 were pipeted into 100 x 12 mm tared tubes (T_1). The tubes were covered with marbles and heated at 97 C for 10 min. After cooling in tap water for 5 min, the exterior of the tubes was wiped with filter paper, the marbles were removed, and the tubes weighed (T_2). They were centrifuged 10 min at 1000 x g then weighed (T_3) after inverting over absorbing paper to drain for 10 min. The water holding capacity was expressed by the formula: $100(T_3 - T_1)/T_2 - T_1$.

Whipping

Solutions of 5% protein in distilled water were made with egg white solids and cottage cheese whey protein isolate. The pH of each sample was adjusted to 9.0 with a saturated solution of $Ca(OH)_2$. Samples were whipped in a Hamilton Beach mixer for 20 min at full whipping speed. Foam stability was determined by measuring drainage through a wire screen at definite intervals.

Textural Properties

Textural properties were measured with an INSTRON texturometer Model 1130. Protein solutions were prepared with distilled water in 3.5 cm diameter beakers and adjusted to pH 7.0 with 1 N HCl or 1 N NaOH. After steaming for 10 min and cooling in tap water for 5 min, the gels were removed with a spatula and cut to make a 2.5 cm high slice. Each gel was compressed 1.5 cm, chewing it in the instrument two times at drive speed 25.4 cm/min, chart speed 25.4 cm/min, and force range 45.45 kg full scale. All runs were in triplicate. Hardness, cohesiveness, and springiness were measured (20) from the recorded curves. Chewiness was expressed as the product of hardness, cohesiveness, and springiness, and gumminess as the product of hardness and cohesiveness.

Replacement of Egg White in White Cake and Taste Panel Evaluation

White cakes were prepared by mixing: 147 g bleached enriched white flour, 173 g granulated sugar, 63 g hydrogenated shortening, 143 g reconstituted nonfat dry milk, 9.2 g of whey protein isolate or dried egg white, 58 g of tap water, 6.0 g double acting Calumet baking powder, 4.0 g of salt, 3.0 g imitation vanilla. The whey or egg white was hydrated before use. The whey cake received an addition of .64 g of an emulsifier blend prepared by mixing 75.4 g water, 17.1 g Span 60 (Sorbitan Monostearate), and 7.5 g Tween 60 (polysorbate 60).

Samples of each cake were submitted to 24 educated panelists who were asked to rank them in order of preference. The ranked sum was determined by assigning a number 1 to the cake listed first and a number 2 to the cake listed second by each panelist.

RESULTS AND DISCUSSION

Addition of polyacrylic acid to a cheese whey solution adjusted to pH 3.8 to 4.2 gives a white precipitate of protein-polyacrylate. The large particles of precipitate coated filter aid have the tendency to settle fast without sticking to each other, leaving a translucent supernatant. In approximately 1 h, the precipitate shrinks to a minimal volume, the supernatant can be syphoned, and the remaining solids representing about 30% of the initial volume, can be filtered, or centrifuged (Fig. 1). Calcium carbonate can substitute the more expensive magnesium carbonate in the reaction with protein polyacrylate. However, should the protein be used for soft drinks fortification the calcium may precipitate with citric acid forming insoluble deposits of calcium citrate. The reaction with magnesium carbonate regenerates the protein and precipitates the polyacrylic acid as magnesium polyacrylate. The volume of water used for the reaction represents 1/5 of the initial whey volume; thus, the protein concentration is higher than in the whey, and drying

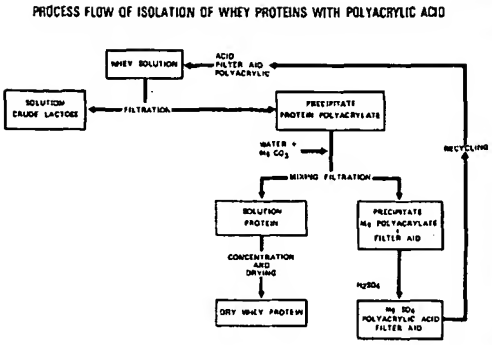

FIG. 1. Process flow of isolation of whey proteins with polyacrylic acid.

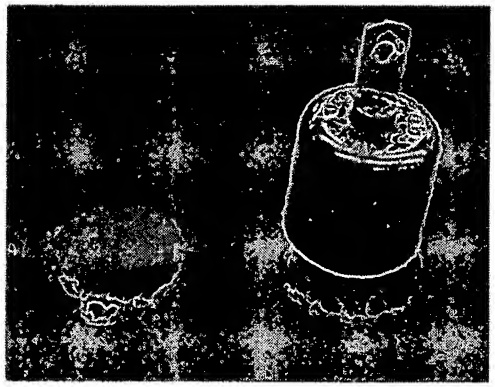

FIG. 2. Steam coagulated cottage cheese whey protein isolated with polyacrylic acid. Solution of 10% wt/wt steamed 3 min. The weight was 295 g.

costs of the finished product are reduced.

The cake of magnesium polyacrylate and filter aid is treated with dilute sulfuric acid which transforms it into a solution of polyacrylic acid and magnesium sulfate containing insoluble filter aid. This stream is recycled into the process to precipitate the protein from the next batch of whey. If whey batches have the same size, supplementation with 10% fresh polyacrylic acid into the recycled polyacrylic acid stream insures optimal recovery of protein. The magnesium sulfate which results from the treatment of magnesium polyacrylate with sulfuric acid and any excess of polyacrylic acid are purged into the lactose stream where the latter can be decreased by liming and filtration as insoluble calcium polyacrylate.

The protein recovery figures from cottage cheese or cheddar cheese whey, based on protein in the initial whey which can be precipitated in 12% trichloroacetic acid, were 85.7 and 86.7%, respectively. However, the recovery of total nitrogen was only 58.7% in cottage cheese whey and 63.3% in sweet cheese whey (Table 1) due to nonprotein nitrogen which does not precipitate with polyacrylic acid. The differences between the total nitrogen recoveries of the two whey varieties are due to higher nonprotein nitrogen values in the cottage cheese whey compared to the sweet whey. Peptide fragments resulting from microbial or rennet proteolysis of milk proteins, such as the κ-caseinoglycopeptide, do not precipitate with polyacrylic acid (21) because of molecular size or carbohydrate hindrance. Because cottage cheese whey contains a higher ratio of nonprotein nitrogen to total N than sweet cheese whey, the recovery of nitrogen is higher in the supernatant of cottage cheese whey compared to the same step in sweet whey. Inversely, the

TABLE 1. Recovery of total nitrogen from cottage cheese whey and sweet whey. Results in percent in each step vs. initial whey nitrogen.

Process step	Type of cheese whey	
	Cottage cheese[a]	Sweet whey[b]
Supernatant of polyacrylic precipitation	37.8	31.6
Protein, polyacrylate precipitate	62.2	68.4
Precipitate of Mg polyacrylate and filter aid	2.0	3.6
Unaccounted losses	1.5	1.5
Total recovered nitrogen in dried products	58.7	63.3

[a]Total N: .100%; Nonprotein N: .028%.

[b]Total N: .121%; Nonprotein N: .026%. Averages of five batches.

TABLE 2. Protein composition of whey protein preparations. Results in percent of total protein.

Protein fraction	Polyacrylic acid precipitated		Ultrafiltered protein
	Cottage whey	Sweet whey	
β-Lactoglobulins	72.3	69.8	50.3
α-Lactalbumin	16.1	12.0	22.4
Proteose peptone	2.3	3.3	12.4
Bovine serum albumin	3.3	7.8	4.8
Immunoglobulins	6.1	7.3	10.2

recovery of nitrogen in the precipitation step is higher when sweet whey is used as the raw material instead of cottage cheese whey. The behavior of the two types of whey during processing was otherwise identical, widening the scope of the method to more than one source of raw material.

The selective precipitation of proteins is emphasized by the electrophoresis results (Table 2). While the protein composition of both sweet and cottage whey preparations is similar, the proteose-peptone content is, respectively, 3.7 and 5.3 times smaller in the two polyacrylic precipitated samples than in a sample of ultrafiltered whey.

Noticeable in the proximate analysis of the finished product (Table 3) are the high protein content (80 to 84%) and low figures for nonprotein nitrogen, lactose, fat, and lactate.

In the mineral analysis of the whey protein isolate (Table 4), magnesium accounted for 76% of the determined cations followed by potassium. Most of the magnesium is introduced in the solubilization step of the protein polyacrylate complex.

TABLE 3. Proximate analysis of protein isolated from cottage cheese whey. Value ranges determined on 12 batches.

	%
Moisture	5.7 to 6.8
Nitrogen	12.6 to 13.6
Nonprotein nitrogen	.05 to .07
Protein (N X 6.38)	80 to 84
Fat	.4 to 1
Lactose	3.3 to 6.5
Ash	.9 to 13.0
Lactate	1 to 1.6
Polyacrylic acid	<.001

The averaged compositions of five batches of lactose streams obtained by the described procedure with recycling of polyacrylic acid are given in Table 5. Compared on total solids basis to data reported on streams resulting from reverse osmosis isolation of cottage cheese whey proteins (18), the streams of lactose from the present process contain more lactose, twice as much lactic acid, and 62 times more nitrogen. Though variations of whey samples used in the two isolation methods may account for some of the observed differences, the selectivity of polyacrylic precipitation of proteins must play an important role, rejecting into the lactose stream nonprotein materials and nonprotein nitrogen.

The nutritional value of whey proteins isolated from cottage cheese sweet whey (Table 6) is reflected by the composition of essential amino acids. Compared to the recommendation of the Food and Agriculture Organization of the United Nations (4), all essential amino acids are in adequate supply.

The solubility of a water solution of 1% protein isolated from cottage cheese whey is independent of the pH between pH 3.0 and 9.0. Within these limits of pH, virtually all of the

TABLE 4. Mineral analysis of protein isolated from cottage cheese whey.

	%
K^+	.1820
Na^+	.1190
Ca^{++}	.0875
Mg^{++}	1.27
Fe^{+++}	.0064
PO_4^{3-}	2.1
Cl^-	.3

TABLE 5. Proximate analysis of total solids in the lactose stream resulting from cottage cheese protein recovery with polyacrylic acid.

Determination	%
N	.62
Lactose	73.82
Lactate	11.17
Fat	.99
Ash	13.50
Cations in ash of lactose stream (in g/100 g ash)	
Na	7.4
K	21.3
Ca	23.3
Mg	5.2

isolated protein is soluble. This range covers protein fortified beverages, pointing to potential applications of the product in nutritional beverages. At pH 6.5, concentrations close to 10% (wt/vol) can be obtained by dissolving the dry protein preparation in water. However, the solubility of the whey protein preparation tends to decrease at higher concentrations (Table 7). The proteins which remain insoluble after mixing 15% protein in water contain more β-lactoglobulins and less α-lactalbumin than the sample used to prepare the solution, indicating solubility differences among individual proteins of the mixture. Separation of the insolubles by centrifugation and subsequent dissolving in water proved that they were not denatured proteins.

The whipping properties were compared with dried egg white (Table 8). Foam volume and drainage of cottage cheese whey protein were close to egg white; however, the heat stability of the foam was much lower.

TABLE 7. Solubility at pH 6.5 of cottage cheese whey protein isolate.

Grams protein added/ 100 ml solution	% Protein in solution
1.0	1.0
5.0	5.0
10.0	9.4
15.0	12.6
20.0	15.4

A property of proteins isolated from cottage cheese or sweet whey by the method of polyacrylic precipitation is the ability to heat coagulate into firm structures similar to boiled or dried egg white (Fig. 2). A study of the coagulation properties of the whey protein isolate, compared in some instances to egg white, was deemed necessary to evaluate possible replacement of egg whites in food applications.

The coagulation temperature at pH 7.0 of a 16% cottage cheese whey protein solution is 68 C compared to 61 C for egg white determined in the same conditions.

Least coagulable protein concentration (Fig. 3) for the whey protein isolate is more dependent on the pH than that for egg white. However, between pH 6 and 7, frequently encountered in food systems, the two curves follow a similar course. The irregular shape of the whey protein curve may be viewed as the

TABLE 6. Essential amino acids of whey proteins. Results expressed in g amino acid/100 g of protein.

Amino acid	Cottage cheese whey protein isolate	Sweet whey protein isolate	FAO[a]
Isoleucine	4.7	4.6	4.2
Leucine	14.9	11.5	4.8
Lysine	12.0	9.6	4.2
Methionine + cystine	5.9	4.8	4.2
Phenylalanine + tyrosine	8.1	6.6	5.6
Threonine	5.9	5.1	2.8
Tryptophan	1.8	not determined	1.4
Valine	4.6	3.9	4.2

[a]Recommendations of the Food and Agriculture Organization of United Nations (4).

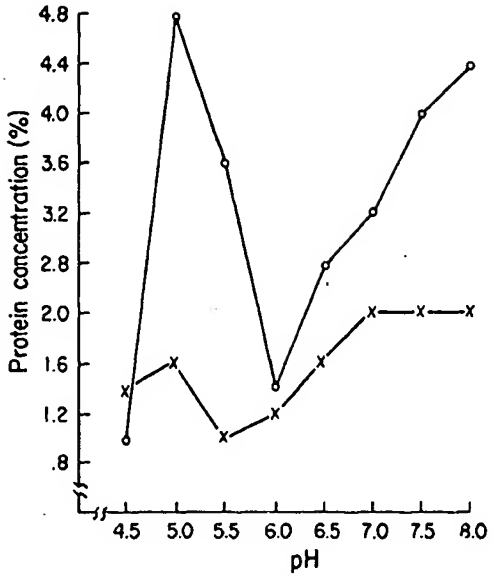

FIG. 3. Least coagulable protein concentration at different pH values. Egg white x - x, cottage cheese whey protein isolate 0 - 0.

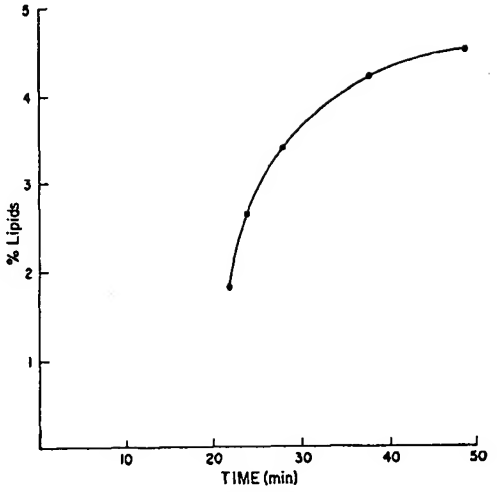

FIG. 4. Relationship between coagulation time and lipid concentration in 10% (wt/wt) cottage cheese whey protein.

result of the behavior of individual components and their interactions with each other. Thus the increased coagulation at pH 4.5 could be attributed to an association of α-lactalbumin (7) at low pH. Increasing the pH reduces this association, but further increase of the pH toward the isoelectric points of β-lactoglobulins and immunoglobulins lessens electrostatic repulsions favoring aggregation and coagulation. Above pH 6, electrostatic repulsions and dissociations of β-lactoglobulin B into monomers (13) become prevalent decreasing coagulation ability.

At 68 C, the coagulation time decreases from 18 min to 13 min, as the protein concentration increases from 8 to 20%. Protein solubility, kinetic dependency of coagulation on concentration, and increased proximity of solute molecules favoring gel structure formation may be elements of this relationship.

Variations of coagulation time of individual batches of cottage cheese whey protein isolate prompted the study of the effect of lipid concentration. Addition of lipids extracted with methanol-chloroform from dried whey protein isolate into solutions of the protein product increased the coagulation time (Fig. 4). The nature of linkages binding the polypeptide chains in a gel network has been attributed to SH groups reacting to form SS bridges (22) and to intermolecular hydrogen bonds, probably between side chain amino and carboxyl groups (14). Competition of lipid components for the hydrogen binding sites may interfere with formation of the aggregates increasing the time

TABLE 8. Drainage volumes of protein foams.

Product	Foam weight	Foam volume	Drainage (ml) 15	30	60 min
	(g)	(ml)			
Egg white solids	198.8	2260	18	60	105
Cottage cheese whey protein isolate	202.0	1850	20	79	138

TABLE 9. Water holding capacity (%) of egg white and cottage cheese whey protein isolate.

pH	Egg white	Cottage cheese whey protein isolate
6.0	98.2	91.9
7.0	98.8	95.6
8.0	99.4	96.5
9.0	98.4	90.2

TABLE 11. Taste panel results of white cake prepared with egg white solids or cottage cheese whey protein.

Palatability characteristics	Ranked sum
Flavor	
Cake prepared with cottage cheese whey protein	33
Cake prepared with egg white	33
Texture	
Cake prepared with cottage cheese whey protein	35
Cake prepared with egg white	34
Overall acceptability	
Cake prepared with cottage cheese whey protein	33
Cake prepared with egg white	35

necessary to set the coagulum.

The water absorption capacity of coagulated cottage cheese whey proteins was slightly lower than that of egg white (Table 9). However, the former still retained 90 to 96% of the entrapped water after 10 min centrifugation at 1000 x *g*.

Texture measurement data are in Table 10. At 10% protein concentration, the whey protein gel has similar textural properties to the 15% egg white gel. As the protein concentration increases, springiness tends to decrease while hardness and gumminess increase.

To evaluate an actual application of total egg white replacement in a food, white cakes baked with whey protein or egg white were compared. In the first trial, the volume of the whey protein cake was somewhat decreased but this was corrected through the addition of a small amount of surfactants. The ratings for flavor, texture, and overall acceptability were considered equal by a taste panel evaluation (Table 11).

The characterization and evaluation of functional properties of cheese whey proteins recovered by polyacrylic precipitation indicate that the method has distinguishing features from other isolation procedures. The process yields a material with high protein concentration, low lipids, lactose, and very little residual precipitating reagent. Solubility data of the whey protein have confirmed the conclusions of a previous work with enzymes (23) that the recovery of proteins with polyacrylic acid can be done with minimal denaturation.

With regard to the food safety, polyacrylates have been approved by the Food and Drug Administration for several food related uses, but do not have blanket approval for all food applications. Any new industrial application of polyacrylic acids for food uses will be subject to clearance. The fact that only small amounts of residual polyacrylic acid are left in the whey protein at the end of processing is beneficial from the standpoint of food safety.

Increased purification and low protein denaturation, two characteristics of the polyacrylic

TABLE 10. Measures of texture of egg white and cottage cheese whey protein isolate.

Proteins	Textural properties				
	Hardness	Cohesiveness	Springiness	Chewiness	Gumminess
Egg white (15% protein gel)	.49	.58	.48	.14	.28
Cottage cheese whey protein isolate					
10% Protein gel	.52	.60	.65	.20	.31
12.5% Protein gel	.66	.59	.50	.19	.39
15% Protein gel	1.73	.61	.47	.50	1.06

isolation method, may be the determinants of a unique functional property, that of heat coagulation into firm gels. This expands the range of potential applications of whey protein isolated with polyacrylic acid beyond nutritional and solubility into the area of egg white replacement.

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